STUDIES IN THE PHOTOCHEMISTRY OF SOLID CHLOROPHYLL.

Thesis for the degree of Doctor of Philosophy,
presented by

Brian Sunners, B.Sc.

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University of Edinburgh.

C O N T E N T S

INTRODUCTION

It has been known since the end of the eighteenth century (1) that the intake of carbon dioxide and water vapour by plants takes place only in the green parts of the tissue. Thus it followed that the subsequently discovered process of photosynthesis - the production of carbohydrates from these gases - can take place, at least in the initial stages, only in these green parts. The pigment, or pigments, giving rise to the green colour became known as chlorophyll, and were correctly assigned the important property of initiating the photosynthetic process.

Isolation and examination of the colouring matter reveals two types of pigment: (a) the green or chlorophyll fraction, and (b) the yellow or carotene fraction. The function of the latter pigments is still uncertain; it may be that of photosensitisor (2), making available to the chlorophyll wavelengths of light not directly absorbed. Chemically, the carotenes are long chain polyenic compounds of molecular weight approximately 600; whereas the chlorophyll fraction consists principally of two very similar highly substituted

derivatives of dihydroporphyrin, or 'chlorin', molecular weight 900. It is this latter fraction that is responsible for the initial stage of photosynthesis, the transfer of radiant energy to the chemical system, thus enabling the reaction to proceed against the energy gradient.

Chlorophyll is found with only very slight structural variations in all forms of plant life, from the very highest and most complex systems to the simplest algae and photosynthetic bacteria, and may therefore be justly described as probably the most important organic compound contributing to life on the earth.

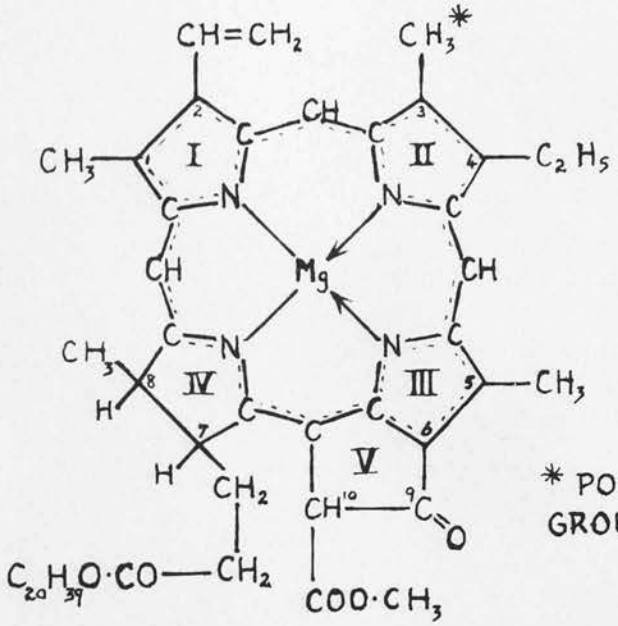
A great deal of investigation has naturally centred around these pigments, and many attempts (3) made to reproduce photosynthesis in vitro. These attempts are now all considered to have been unsuccessful, although at the time many claims for the artificial production of carbohydrates were put forward.

The overall reaction for photosynthesis may be represented by the equation:-

$xCO_2 + xH_2O \xrightarrow[\text{chlorophyll}]{\text{light}} (CH_2O)_x + xO_2$, where $(CH_2O)_x$ represents the various carbohydrate products. This is an oxidation/reduction system catalysed by the chlorophyll, which may act either in the reduction of the CO_2, or by splitting the water molecule into O and OH. The latest work (4) shows that the actual process occurring is the reduction of the CO_2, but that it is not carried out by the pigment directly but by a complex biochemical system, for which the energy is supplied from sunlight by the chlorophyll. The possibility that the chlorophyll is directly concerned with the splitting of the water is not yet ruled out.

Thus much of the previous work on chlorophyll has been directed to an extensive investigation of the oxidation-reduction properties, and many of the results form today not only the basis of our knowledge of chlorophyll chemistry, but also contribute extensively to the general theory of other dye and pigment systems.

The presently accepted structure of chlorophyll is that put forward by Fischer in 1939 (5) (Fig. 1). The



* POSITION of —CHO GROUP IN CHLOROPHYLL 'B'

FIG. 1

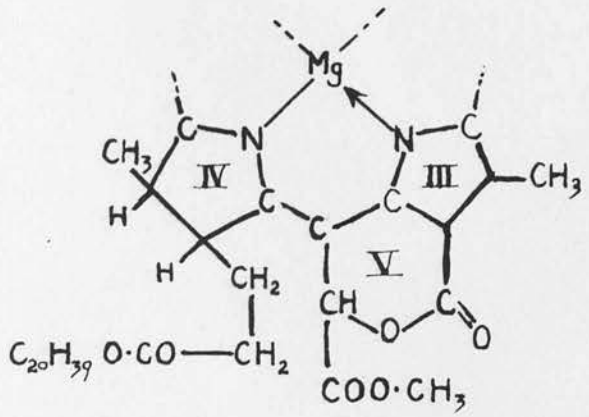


FIG. 2

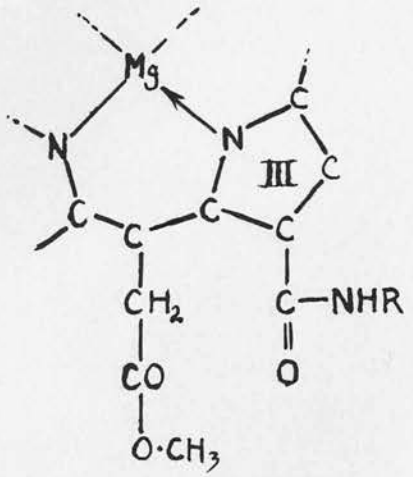


FIG. 3

dotted line indicates the conjugated ring of the aromatic nucleus. The positions of the N-Mg bonds is not definitely known, though molecular orbital considerations (6) favour the above variation. The other possibilities may be responsible for the tautomeric or isomeric forms known as chlorophylls a', b' (7), and chlorophyll 'X' (8), at present otherwise unidentified. This requires the magnesium to be unsymmetrically placed with respect to the four nitrogen atoms, a condition not favoured by the size factors, thus the structure may be mesomeric. The C_{20} side chain is the esterified phytol group.

Attempts to reduce chlorophyll have been concerned principally with the formation of a reversible reduced 'leuco' (colourless) form (9); but although this is partly possible by reversible hydrogenation of the porphyrin ring, the vinyl group on C_2 is irreversibly reduced, so that the re-oxidised product is never identical with the starting material, even though the colour appears to be completely restored. The only claim for a truly reversible reduction was put forward

by Krasnowski in 1948 (10), using ascorbic acid in pyridine solution; here also, however, there may be room for doubt, as spectroscopic differences between the starting material and product have been detected.

There are two points in the above molecule that are particularly susceptible to oxidation, the two hydrogen atoms on ring IV, and the lone hydrogen atom on C_{10}. The hydrogenated bond in ring IV is responsible for the characteristic absorption peak at about 6600 Å in all chlorins, and so its behaviour can be closely followed. This bond can be easily oxidised; the loss of the two hydrogens may even occur by an internal oxidation/reduction mechanism in a reduction reaction (11).

Also important are the reactions of the hydrogen atom on C_{10}. The keto-enol couple formed by this group and by the neighbouring carbonyl group is responsible for many of the characteristic reactions of chlorophyll, of which perhaps the most well known is the Molisch phase test (12). In this test, an alcoholic solution of an alkali is added to an ether solution of chlorophyll, and a transient brown phase appears between the two liquid

layers; this distinguishes pure chlorophyll from 'allomerised' samples, which do not give the reaction. The brown phase is believed to be due to salt formation between the alkali and the enolised carbonyl group in the cyclopentanone ring; finally the whole of this ring is ruptured by the alkali and the final green product, a chlorin, is formed.

A second reaction involving this hydrogen atom is the 'allomerisation' (13) of chlorophyll, whereby alcoholic solutions of chlorophyll when left exposed to the air take up one molar proportion of oxygen (14), with the formation of a chlorin lactone (15) (Fig. 2). This is the same lactone as is formed in the phase test; allomerisation is therefore just a slow phase test in which the brown phase does not appear. Alcohol is always necessary, even in traces, but the oxygen may be replaced by quinone, when a 10-methoxy compound is formed.

The C_{10} position is also concerned in the reaction between chlorophyll and amines (16), involving the formation of chlorin-6-acid amides. In this case no oxygen is required. The phosphorescence of chlorophyll

in amine or amine/hydrocarbon solutions is due to this compound (Fig.3).

Probably the most important oxidation reactions of chlorophyll are the bleaching reactions; both reversible and irreversible. The most obvious example of irreversible bleaching occurs in autumn leaves, but this may be demonstrated in young leaves by inhibition of photosynthesis, or by photosynthesis in a high concentration of oxygen. Solutions of chlorophyll bleach on exposure to light, but not if oxygen is rigorously excluded (17). Films of solid chlorophyll have been shown to take up oxygen when bleaching under illumination even more rapidly than in solution (18). It is for these reasons that irreversible bleaching is believed to be an oxidation reaction. Quantum yields in solution, however, suggest that some if not all of the oxygen taken up is transferred to the solvent or to an impurity.(19).

Reversible bleaching occurs only to a slight extent on illumination in red light; with about 1% decrease in absorption (20). The original intensity of colour is restored in dim light, provided oxygen is absent. If

it is present, then the reaction is merely inhibited, and not made irreversible. Blue light degrades the pigment, presumably by breaking down the unstable excited molecule formed on illumination. This phenomena is found in other pigments, some of which, where restoration of the colour is very slow, can be completely and reversibly decolourised. Kinetic measurements show that this is probably an oxidation/reduction reaction with the solvent or an impurity, and not a tautomerisation or dismutation between chlorophyll molecules.

Complete reversible bleaching of chlorophyll dissolved in methanol can be brought about by the addition of some highly charged inorganic ions, e.g. Fe^{+++}, Ce^{++++}, causing the solution to turn yellow. The colour can be restored completely by ferrous ions, suggesting an oxidation/reduction reaction between the chlorophyll and the ferric ions. This is supported by the fact that ferrous ions can be detected in bleached solutions. Again, the bleached form of the pigment is unstable in blue light, and the reaction becomes irreversible. As this reaction can be carried out with allomerised chlorophyll, it cannot be occurring at C_{10}.

Thus there is a considerable amount of evidence for the existence of one or more oxidised forms of chlorophyll, reversible or otherwise, depending on the conditions of the experiment. The actual nature of this form is very much in doubt, however, particularly as the part played by the solvent in all these cases is unknown. An attempt was made in 1914 to overcome this difficulty by bleaching solid films of chlorophyll supported in gelatine or collodion in an atmosphere of oxygen; beyond reporting an uptake of oxygen, however, these earlier workers made little attempt to elucidate the mechanism of the reaction, the work being principally concerned with the nature of the organic products.

A property of chlorophyll which has received some attention in recent years is its alleged deodorising action. No direct evidence could be obtained for this, although the action seemed satisfactory in practice. It has recently been shown that chlorophyll, chlorophyllides, or chlorophyllins do not absorb directly any of the common noxious gases (21), but that these compounds do inhibit their formation in the principal putrefaction bacteria (22), e.g. Proteus Vulgaris, by interference with

the metabolic processes of this and similar organisms, thus giving rise to the belief that the chlorophylls act as deodorisers. The action is therefore neither physical adsorption nor direct chemical combination with the gases in question.

In common with many other dyes, chlorophyll possesses the ability to photosensitise the reactions of other molecules; i.e. to enable a reaction to proceed in the presence of chlorophyll when otherwise it would not. The mechanism is essentially one of energy transfer, thus differing from simple catalysis, in which a reaction proceeds through alternative stages each of lower activation energy than the uncatalysed reaction, and thus may proceed faster. The absorption of light by the sensitisor results in the formation of an excited molecule, this may subsequently transfer some of the radiant energy, probably in the form of an excited electron, to the reaction system, providing one component will act as an electron acceptor; more frequently, the electron may be transferred to molecular oxygen to form O_2^- (23), which then reacts in the oxidation of the substrate. In this way is made possible the oxidation of many organic compounds in solution

by molecular oxygen, or more general oxidation/reduction reactions utilising other, and usually more specific, oxidants. Examples of these two types of reaction are (a) the oxidation of allyl thiourea by molecular oxygen in acetone solution (24), also of hydroquinone (25), and (b) the oxidation of phenyl hydrazine by methyl red in the total absence of oxygen (26). In this latter case, the methyl red is not reduced by allyl thiourea, hydroquinone, or other common organic reductants.

Photosensitisation with a solid photosensitisor, as for example the photosensitised oxidations used in this work, proceeds in exactly the same way: by virtue of the photoconducting properties of the sensitisor, an excited electron is transferred to the adsorbed reactant; there is a greater similarity to heterogeneous catalysis here, however, as adsorption may involve molecular strain and thus lower the activition energy of the reaction.

Photosensitisation, and indeed all the more general reactions of dyes and pigments, may be better understood by consideration of the energy changes taking place in a molecule when light is absorbed. The electronic energy levels of a molecule may be represented by a Jablonski

line diagram (Fig. 4). The normal ground state of any molecule (S) is almost invariably a singlet level, i.e. all the electrons have coupled spins. Absorption of a quantum of light increases the energy of one of the orbital electrons so that it may occupy a higher energy level, the first excited singlet level (S_1). Further absorption of light may result in excitation to the second singlet level, to the third and possibly finally in the ejection of an electron from the molecule. Normally, however, the energy is dissipated from the molecule very rapidly by one of a number of processes - collisional deactivation, internal degradation to heat, or quenching by a foreign molecule, a process explained in more detail below. When the upper electronic levels are particularly stable, as in aromatic molecules containing a number of conjugated double bonds, then the life of the excited electron may be sufficiently long, about 10^{-8} sec., to permit the re-emission of the energy in form of light. This process, known as fluorescence, is represented in the diagram by arrows 1 and 2, and is closely connected with the ability of a molecule to photosensitise reactions. It does not follow though that a nonfluorescent substance does not have this property as was originally thought, as

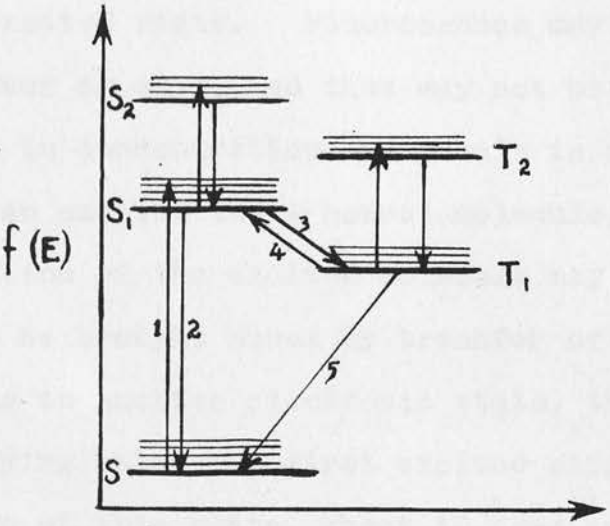
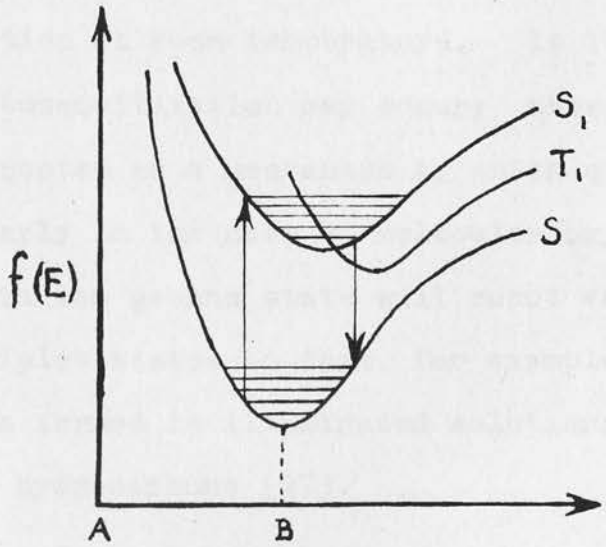


FIG. 4



INTERATOMIC
DISTANCE A-B

FIG. 5

the ability to fluoresce depends largely on the life-time of the excited state. Fluorescence may also be quenched in a number of ways, and thus may not be obvious. An increase in concentration may result in dimer formation between an excited and a normal molecule, or actual dissociation of the excited molecule may occur. Quenching may also be brought about by transfer of the e xcited electrons to another electronic state, the first triplet level, lying below the first excited singlet level; the long life of this state, about 10^{-3} sec. under normal conditions, makes it very susceptible to collisional deactivation at room temperature. It is from this state that photosensitisation may occur; these reactions may also be quoted as a mechanism by which quenching occurs, particularly in the case of molecular oxygen, which being triplet in the ground state will react very readily with other triplet states to form, for example, the transannular peroxides formed in illuminated solutions of many polycyclic aromatic hydrocarbons (27).

When the emission of absorbed radiation continues for a length of time considerably greater than the life-time of the excited state, the phenomenon is commonly known as

phosphorescence (35), and may be of two kinds. The delay in emission of absorbed energy is due to the presence of the triplet energy level lying just below the first excited singlet level (line 'T_1'). The electrons become trapped in this level and thus radiation in returning to the ground state is delayed; the effect is naturally most noticeable at low temperatures or in rigid solvents.

If the electron is thermally raised to the S_1' level, return to ground state shows exactly the same emission spectrum as in fluorescence, and the process is distinguishable only by the time factor, this is α-phosphorescence; the forbidden return direct to the ground state (arrow 5) shows β- phosphorescence, having a unique emission spectrum for each substance.

The identity of this metastable energy level remained unknown for a long time, and was sometimes thought to be a tautomeric state (28). The properties of phosphorescence, however, indicated that a triplet state was more likely (29), i.e. an electronic state in which the spin of one electron had been reversed, and a pair of electrons now have parallel or uncoupled spins. The long life of this state makes it probably the most active

photochemical state, and its presence explains many former anomalies; such as the lack of competition between fluorescence and photochemical reactions or photosensitising power, as would occur if these reactions proceeded from the first excited singlet level. Measurement of the paramagnetism of the phosphorescent state has shown that it is indeed a triplet state (30).

The Franck-Condon diagram (Fig. 5) shows the conditions governing the interchange of the various electronic states more clearly. Although the two dimensional figure above is valid strictly only for diatomic molecules, the same conditions are applicable to polyatomic molecules, in which case the lines above would represent only a section of the multidimensional hypersurfaces necessary to illustrate the motions of the electrons. Curves S, S_1, and T_1 represent the variation in inter-atomic distance with increase in potential energy, (the horizontal lines representing the discrete vibrational levels) for the ground state, the first excited singlet state, and the first triplet state respectively. Excitation of an electron from the ground state is represented by arrow 1; the reverse process, re-emission of fluorescence, is represented by arrow 2;

where curve S_1' intersects curve S, then internal degradation of the energy is possible, and the substance is non-fluorescent. Molecules possessing the third curve C have the ability to switch electrons from the first excited level to this new mode of vibration; this is the triplet level, containing two electrons with uncoupled spins, and so return to the ground state from this level is 'forbidden'. The diagram shows why fluorescence is usually of longer wavelength than the absorbed radiation: the upper excited level corresponds to a weaker i.e. a longer bond, the electron changes states (ideally) only at the extremity of a vibration and thus arrives at a high vibrational level but rapidly falls to the lowest, then back to the ground state emitting the longer wavelength fluorescent light. Fluorescence of the same wavelength as the absorbed light is known as "resonance radiation" and would be represented by arrow 1 reversed in the diagram.

Thus to define completely the action of illuminated chlorophyll, it is necessary to show the existence of a phosphorescent state. Chlorophyll fluorescence has been examined in some detail, the only feature of note being that in pure dry hydrocarbons, the quantum yield is

abnormally low (31), about 0.01%; the usual figure of nearer 10% being attained only when a polar impurity - water, certain alcohols, etc., is added to the solution. This would suggest that the fluorescing species is a chlorophyll - polar solvent complex, and not the individual chlorophyll molecules. Initially it was thought that chlorophyll in amine solutions was phosphorescent (32), but this was shown to be due to compound formation, the chlorin 6 acid amide described above. A claim for the phosphorescence of chlorophyll itself was put forward in 1948 (33), but this was not confirmed; more recently, more evidence has been put forward (34), so that it does appear likely that chlorophyll does have a phosphorescent state, i.e. a triplet state, and that it is from this state that the secondary acts of a photochemical reaction take place, the primary acts being the absorption and immediate conversion of radiant energy.

This is therefore the metastable or tautomeric state of the earlier workers.

The secondary acts of an excited molecule will naturally depend upon the conditions under which it is illuminated. In solution, these are undoubtedly complicated by the unknown role of the solvent - even in

a simple uptake of oxygen, there is doubt as to what extent the chlorophyll itself is oxidised and how much oxygen is transferred to the solvent or trace impurity. The obvious simplification in this rather basic reaction is to eliminate the solvent and to examine the uptake of oxygen by solid chlorophyll. This is the procedure adopted by the earlier workers, Wager, also Warner (18), in 1914. In view of the waxy nature of chlorophyll - (only recently have the first pure crystals been prepared (36)) - it is necessary to deposit the pigment in an inert support; collodion and gelatine have been used, but it is debatable to what extent they are inert, particularly as the previous work has been principally concerned with the organic products of the reaction.

In 1949, this reaction was studied again in the same way (37) - illumination of solid films of chlorophyll in oxygen - in an attempt to determine the kinetics. As support for the pigment, powdered Winchester or Jena glass was used, or for the convenience of accelerated rates of reaction, finely powdered thallous bromide. The films were illuminated by white mercury light, and pressure changes were followed on a Bourdon gauge (the system will be described in detail later).

Initially, commercial samples of copper-stabilised chlorophyll were used, and the first results indicated that oxygen was taken up in a unit molar ratio to the chlorophyll present. The reaction is not simple, however, as on evacuation followed by re-illumination in the same pressure, an accelerated rate was obtained. This suggests immediately the formation of a reversible peroxide, analgous to such aromatic polycyclic hydrocarbon peroxides as are formed with anthracene (38), rubrene (39), etc., and analysis of the oxidised film showed that there was indeed a peroxide group present in approximately unit molar ratio to the pigment oxidised. Oxidation in the presence of various absorbents, however, showed an increase in the extrapolated oxidation ratio; the extrapolated values were not reproducible, but appeared to tend to five with P_2O_5, and to six with P_2O_5 and soda-lime. Thus the apparent unit molar oxidation ratio is actually the resultant of an uptake of 'n' molecules of oxygen, followed by the release of (n-1) molecules of gases absorbed by the P_2O_5 and soda-lime, where $n \fallingdotseq 5$ or 6. These gases were identified from vapour pressure curves as water and carbon dioxide; acetone was also shown to be present, but it is not clear whether this is a reaction product or comes from the film, as the pigment was deposited from acetone solution. A

mechanism to fit the experimental data was worked out (40).

It has been the intention to extend these results in the present work to natural chlorophyll freshly extracted from plants; to examine in further detail the proposed reaction scheme, by product analysis at various stages, and to investigate some of the difficulties which arose in preliminary experiments with natural chlorophyll, such as the increase in the extrapolated oxidation ratio with time, and the lack of reproducibility in the determination of the amount of water produced at varying extents of reaction.

EXPERIMENTAL METHODS.

The apparatus on which the oxidations were carried out is shown in Fig. 6. A spherical reaction vessel was used, about 30 - 40 mls. capacity, attached to the Bourdon gauge by an A10 ground glass joint. The gauge and its jacket were attached to the main vacuum line, to which were also attached the manometers, a McLeod gauge, the gas storage bulbs, and some other small vessels by ground glass joints to be described in more detail later. The line was evacuated through a cooled trap B to just below 10^{-3}mm. by a two stage Edwards 'Hyvac' oil pump, and below this pressure when necessary by a mercury diffusion pump; to prevent too rapid evacuation the lower line was used initially and only at low pressures was the main line used; this is essential to safeguard the delicate gauge. The apparatus was built entirely of soda glass (with the exception of some special reaction vessels in Pyrex) and all taps were high-vacuum 2 mm. lubricated with Apiezon 'L' grease. Oxygen was admitted directly to the system from the storage bulbs, and dry CO_2-free air was admitted through the air leak. Owing to the difference in the volumes of the gauge and jacket, great care had to be exercised in the admission and

removal of gases to and from the system, as the average gauge is liable to fracture under pressure differences greater than about five millimetres of mercury.

Owing also to this difference in volume, the system must be accurately thermostatted. Gauge, jacket, and reaction vessel were therefore immersed in a glass-sided tank of water kept at 25°C. by means of a lamp heater connected in series with a 200 ohm. resistance, and controlled by a toluene-mercury regulator and valve relay control. By fitting a spiral of thin-walled glass tubing to the regulator (6 x 50 mm. diameter turns of 5 mm. tubing) the temperature fluctuations were reduced to less than 0.005°C. measured at any one point in the tank with a Beckmann thermometer; the temperature gradient along the two foot length of the tank was 0.015°C. The water was circulated around the tank by a small Stuart Turner pump fitted externally. Under these conditions, it was impossible to detect any pressure variations due to temperature fluctuations at a total pressure of up to 300 mm.

Pressure changes in the reaction space were measured by means of a telescope containing a graduated scale,

focussed on the illuminated platinum tip of the gauge pointer. This telescope scale was calibrated in terms of millimetres of mercury as follows:-

The number of scale divisions travelled by the gauge pointer in one complete sweep of the scale is equivalent to a pressure change too small to be detected on the manometer, and so the total number of divisions travelled in several sweeps (counted in one direction only) was recorded as small quantities of gas were added to (or removed from) the gauge. At the end of each sweep, the pointer was brought back to its starting point by adding (or removing) the same pressure of gas to or from the jacket; this also produced what was eventually an appreciable reading on the manometer. The determination was carried out at 300, 100, 50 and 5 mms. absolute pressure, and no variation of sensitivity with pressure was found.

Sample results:-

Total number of divisions	Pressure Change	Sensitivity in mm./div.
276.8	6.6 mm.	0.0238
459.8	11.2 mm.	0.0244
407.6	9.4 mm.	0.0232
345.0	8.4 mm.	0.0243

$\therefore$ Average value of the sensitivity = 0.024 mm./division.

The computed error of this determination is 1%, and the standard deviation of the results is 1.3%.

The total volume of the reaction space was required for certain calculations, and so was determined by slowly expanding air at a known pressure from the reaction space, firstly into the evacuated connecting tubing isolated by taps 1, 2, 3 and 4, on Fig. 6, and then into an evacuated vessel of known volume attached to one of the ground glass joints below a tap 4. The pressure in the jacket was decreased simultaneously to balance the gauge, and the equilibrium pressures were read on the manometer. The volume of the standard vessel was found by weighing it first empty, and then filled with water up to and including the bore of tap 4, before sealing this on to the vacuum line.

Sample results:-

V_1 = volume of the reaction space,

P_1 = initial pressure

V_2 = volume of the connecting lines,

P_2 = equilibrium pressure in $V_1 + V_2$

V_3 = volume of the standard vessel,

P_3 = equilibrium pressure in $V_1 + V_2 + V_3$

Now $V_1P_1 = P_2(V_1+V_2) = P_3(V_1+V_2+V_3)$, and therefore

the unknown V_2 can be eliminated by substituting $V_2 = V_1 \frac{P_1 - P_2}{P_2}$ in $V_1P_1 = P_3(V_1+V_2+V_3)$ giving:

$$V_1 \left(P_1 - \frac{(P_1 - P_2)P_3}{P_2} - P_3 \right) = P_3V_3.$$

Thus V_1, the required volume of the reaction space, is given by:-

$$V_1 = \frac{P_3V_3}{P_1 - \frac{(P_1 - P_2).P_3}{P_2} - P_3}$$

The volume of the calibrated flask was 112.0 mls.; typical pressure readings were:-

P_1	P_2	P_3
757.2 − 487.8	756.4 − 536.8	755.6 − 675.8
=269.4 mm.	=219.6 mm.	= 79.8 mm.

$\therefore$ V_1, reaction volume = 52.12 mls.

P_1	P_2	P_3
755.4 − 460.2	755.0 − 513.8	754.4 − 667.2
=295.2 mm.	=241.2 mm.	= 87.2 mm.

$\therefore$ V_1, reaction volume = 51.80 mls.

Thus the average value of the reaction volume = 51.9 mls.

The volumes of the actual film and any absorbent (when used) were neglected.

The standard deviation of this result is 0.3%.

The substances examined were illuminated with a Mazda 250 watt compact source (ME/D) mercury vapour lamp, housed in a metal box behind the reaction vessel, and cooled by an electric fan (Fig.7). The beam of light, after passing through a 3 cms. aperture, was controlled by a shutter and focussed by a 500 mls. flask filled with water; this acted also as a heat filter. The soda glass of the system removed all U.V. light from the beam, which therefore contained no light of wavelength less than 3650 Å. As an arbitrary check on the intensity of the illumination, a photocell was placed in the light path, together with a neutral filter and protecting shutter, and the photocurrent was measured at intervals of some weeks on a sensitive galvonometer. The useful life of the lamps was found to be a little longer than the specified 500 hours, marked drops in intensities occurring after 600 and nearly 650 hours.

The oxygen used was obtained from a commercial cylinder, and was purified by passing over P_2O_5, and then slowly through two traps cooled in liquid oxygen before storing in two 2-litre bulbs.

A small quantity of pure water was stored in a vessel

attached below a tap at 4 on the figure. It was distilled three or four times between two of these vessels, frozen out at about -40°C. after each distillation, and evacuated with the oil pump to remove any dissolved air.

Two sources of chlorophyll were used, the first a commercial preparation supplied by J. F. McFarlan & Co. of Edinburgh, and the second natural chlorophyll samples freshly extracted from spinach or stinging nettles.

The first sample was purified by shaking sufficient paste in 100 mls. of acetone overnight, filtering, and then diluting the solution to one litre, to give a concentration of about one gram of mixed pigment / litre. A 25 mls. portion of the solution was next evaporated to dryness in a small crystallizing dish, stirred up in a few mls. of 60 - 80° petroleum ether (in which chlorophyll is insoluble) and deposited on top of a bonemeal column 2 x 25 cms. previously washed with about 300 mls. of the same solvent. The carotenes were eluted first by washing the column with between 200 and 300 mls. of petroleum ether, then after the eluate had been running colourless for 50 - 100 mls., the chlorophyll was eluted with A.R. acetone. If natural chlorophyll is purified

by this method, a very slow-moving orange band is found below the chlorophyll; it is more convenient to separate this band completely from the green bands, andremove the chlorophyll by extracting these green bands into acetone, than to attempt to elute each band in turn. As the topmost green band cannot be eluted, nor removed by boiling the bonemeal in several common organic solvents, it is considered to be a strongly adsorbed degradation product. All fractions were filtered after elution, made up to a standard volume, and concentrations and absorption spectra determined as detailed below.

Fresh chlorophyll was extracted and purified by a modification of the method of Zscheile and Comar (41), using pieces of apparatus described by Griffiths and Jeffrey, and also by LeRosen (42).

The procedure finally evolved is as follows:- 250g. batches of shredded spinach, freshly gathered, were extracted in a Townsend and Mercer macerator with 400 mls. of A.R. acetone and a few pieces of crushed ice for five minutes. The extract was filtered and the residue washed with a little more acetone, making the total volume about 500 mls. It is not necessary to add calcium carbonate to spinach extracts to prevent phaeophytin formation., but

in the case of stinging nettles, 1 g. of carbonate was added to every 100 g. of leaves (43). The solution was next added to 250 mls. of diethyl ether in a large separating funnel. Distilled water was added to form two layers, and the funnel was rotated slowly, thus transferring the pigments to the ether layer and at the same time avoiding the formation of the very stable emulsions which occur between water and ether solutions of chlorophyll. The mixed pigments were scrubbed through distilled water using an improved form of the apparatus described by Griffiths and Jeffrey (Fig. 8). The solution of chlorophyll flows by gravity down the capillary tube 1 and up the washing tube 2. It then passes into a large coil cooled with an ice and salt mixture, and finally is collected in the separating funnel 'S', from which any water frozen out of the washed ether solution can be removed. Although Griffiths and Jeffrey state that the solution is now dry enough to pass through the chromatographic columns, it was generally dried overnight over anhydrous sodium sulphate as an extra precaution. The rate of flow of the distilled water down the washing tube is controlled by the height of the syphon tube 3 above the constant level device in

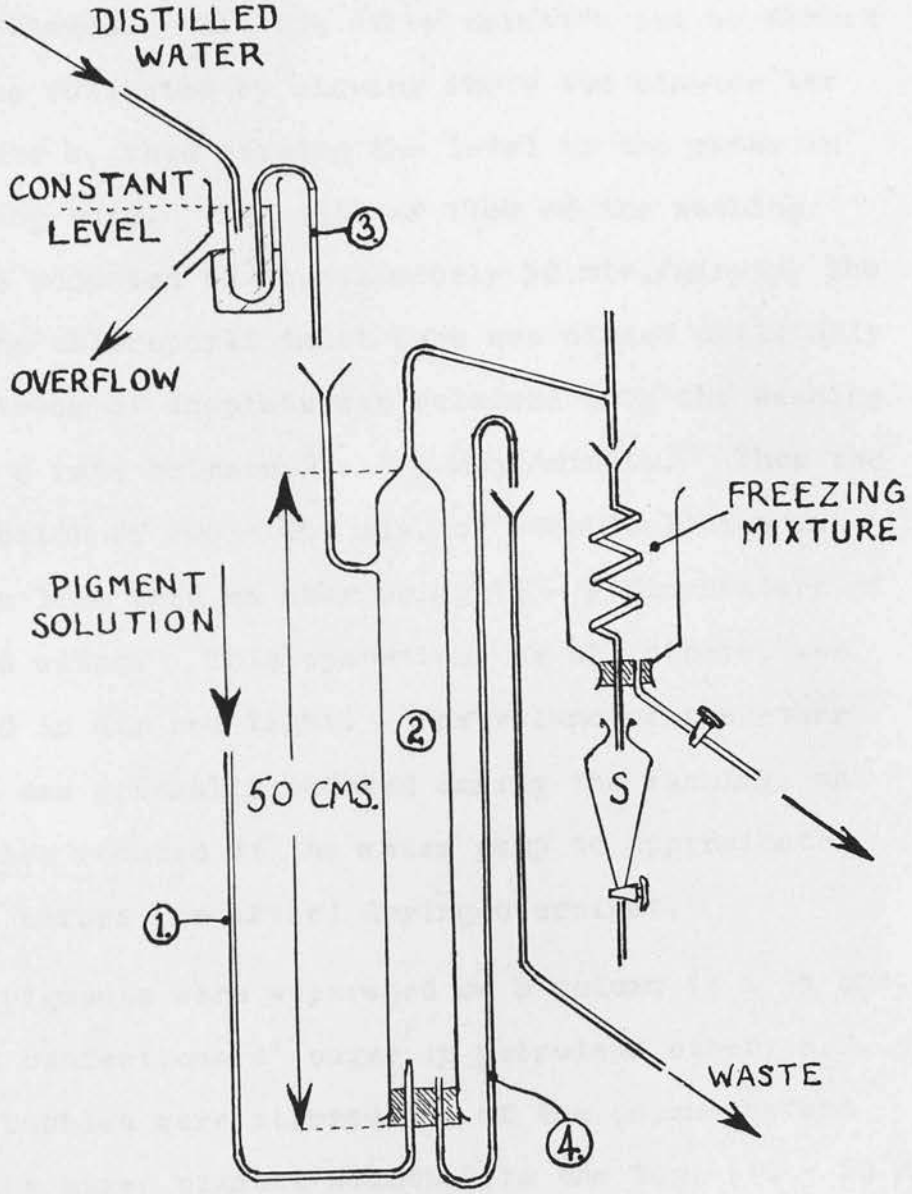

DISTILLED
WATER
CONSTANT
LEVEL
3
OVERFLOW
FREEZING
MIXTURE
PIGMENT
SOLUTION
2
50 CMS.
S
1
WASTE
4

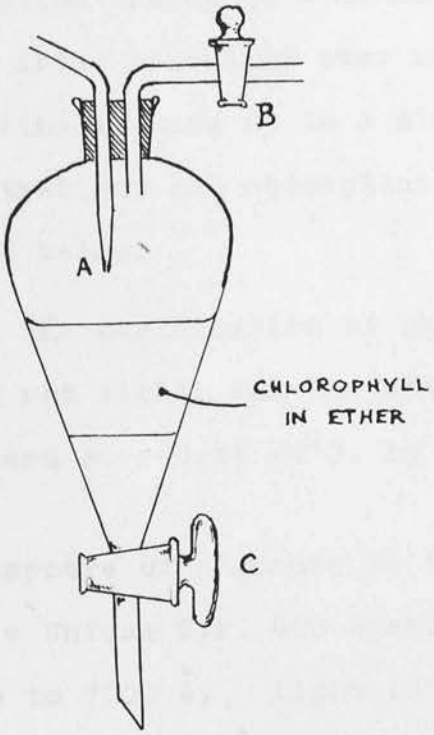
FIG. 8

which it stands. All the ether solution can be washed over to be collected by closing for a few minutes the outlet tube 4, thus raising the level of the water in the washing tube. The rate of flow of the washing water was adjusted to approximately 50 mls./minute; the tip of the chlorophyll inlet tube was closed until only a fine stream of droplets was released into the washing water at a rate between 2 and 5 mls./minute. Thus the average batch of 200 - 250 mls. of ether solution was washed in less than an hour using $1\frac{1}{2}$ - 2 Winchesters of distilled water. This operation, as all others, was performed in dim red light. The volume of the ether solution was gradually reduced during the washing, and was further reduced at the water pump to approximately 100 mls. before (or after) drying overnight.

The pigments were separated on a column (3 x 35 cms.) of dried confectioners' sugar in petroleum ether, B.P. 35-40°C. Any air bubbles were stirred out of the column before adding the mixed pigment solution to the top, (10 - 20 mls.) which was also stirred in to a depth of 10 mms. to ensure even descent of the pigment bands. The carotenes were eluted with about 200 to 300 mls. of petroleum ether, and

then the chlorophyll was eluted with ether. In some cases, it was necessary to elute a slow-moving orange band (probably xanthophyll) with a mixture of petroleum-ether: ether in the proportion 10 : 1 or 8 : 1; alternatively, the band was washed well clear of the green bands, which were then stirred into 50 mls. of ether, and the sugar filtered off through sintered glass. During the development of the columns, the chlorophyll moved slowly down to about half the length of the column and tended to break up into its two components A and B; these were never isolated, however, as previous work has shown that the reaction under investigation appears to be common to both. The separation varied considerably from sample to sample, and even from column to column, and so each had to be treated on its own merits. This is in accordance with the experiences of other workers (44). After elution, the solution was washed free of dissolved sugar using the device described by leRosen, (Fig. 9). Distilled water is run from the fine jet A onto the surface of the chlorophyll solution until the separating funnel was approximately half full; tap B was then closed, C opened so that the washing water ran through the solution continuously, the liquid levels remaining constant. About 1 litre of washing water was used to wash 100 mls.

FIG. 9



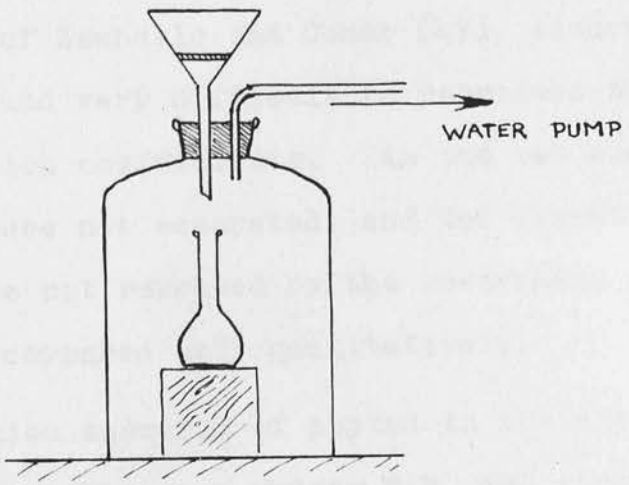


FIG. 11

of solution, the operation taking 30 - 40 minutes. The solution was next dried overnight over anhydrous sodium sulphate, and finally made up to a standard volume and the concentrations and absorption spectra determined as detailed below.

All operations in the purification of chlorophyll were performed in dim red light, and the solutions, crude and purified, were stored at -5°C. in the dark.

The absorption spectra of pigments in the visible region were taken on a Unicam S.P. 600 spectrophotomer, over the range 3500 Å to 7000 Å. Light intensity measurements were made every 100 Å, and 50 Å at the characteristic peaks. The purity of the chlorophyll was determined largely by the spectra, using as a reference those of Zscheile and Comar (47), although it was generally found very difficult to reproduce these authors' extinction coefficients. As the two components of chlorophyll were not separated, and the finest details of the curve were not revealed by the instrument used, the curves were compared only qualitatively.

The absorption spectrum of phytol in the ultra-violet region was taken on a Unicam S.P. 500 spectrophotometer, between 2200 Å and 4000 Å, using spectroscopically pure

cyclohexane as solvent.

The concentrations of the solutions were found by evaporating the solvent off a five or ten ml. sample in a small weighed crystallising dish under water pump vacuum, and then evacuating to constant weight for half an hour at a time at the oil pump through a liquid oxygen trap. All weighings were done on a semi-micro balance, and each determination was duplicated.

Preparation of phaeophytin:- Samples of pure phaeophytin were prepared from chlorophyll by a small scale adaption of the method of Willstatter (45), as follows: 5 mg. of oxalic acid were added to 25 mls. of an acetone solution of purified chlorophyll, concentration about 1 mg./ml. The mixture was shaken in the dark overnight, added the following morning to 10 mls. of a chloroform/water mixture, (1:1), and extracted. The aqueous layer was discarded and the chloroform layer washed with several 5 mls. portions of distilled water until the washings gave no reaction with dilute potassium permanganate. The chloroform solution was dried over P_2O_5 overnight, and then to constant weight at the oil pump. The residue was finally dissolved in a measured volume of acetone, and the absorption spectrum determined in the usual way.

Preparation of ethyl chlorophyllide: This was prepared according to the method of Willstatter and Stoll (46). An 80% alcoholic extraction of hedge parsley leaves prepared in a macerator was allowed to stand in the dark for a few days, while a black waxy precipitate slowly formed. The yield was increased by adding a little aqueous extract, but was still very poor. The crystals were filtered off, and washed extensively with ether. They were in appearance dark and powdery, with an almost metallic lustre, but under polarised light in the microscope were shown to contain some non-crystalline material. A powder photograph, however, confirmed the essential crystalline nature of the product; it could also be precipitated from acetone solution by the addition of water, forming a suspension of microcrystals, easily distinguished from the colloidal suspension of chlorophyll formed under the same conditions.

Method of oxidation:- Two substrates were used to support the chlorophyll, (a) Thallous bromide - this was prepared by adding 50 mls. of thallous nitrate solution (5.32 g. $TlNO_3$ in 100 mls. solution) slowly to 25 mls. of potassium bromide solution (5.00 g. KBr in 100 mls.) contained in a 100 mls. flask, with continuous shaking in dim light. The precipitated thallous bromide was washed

five times with 50 mls. portions of distilled water by decantation, then dried for two days over P_2O_5 in a vacuum desiccator. The powder was next ground, and stored over P_2O_5 in the dark. (b) Jena glass 'J' - the glass was obtained already finely ground, and was stored over P_2O_5. The composition is given in the appendix, p. 105

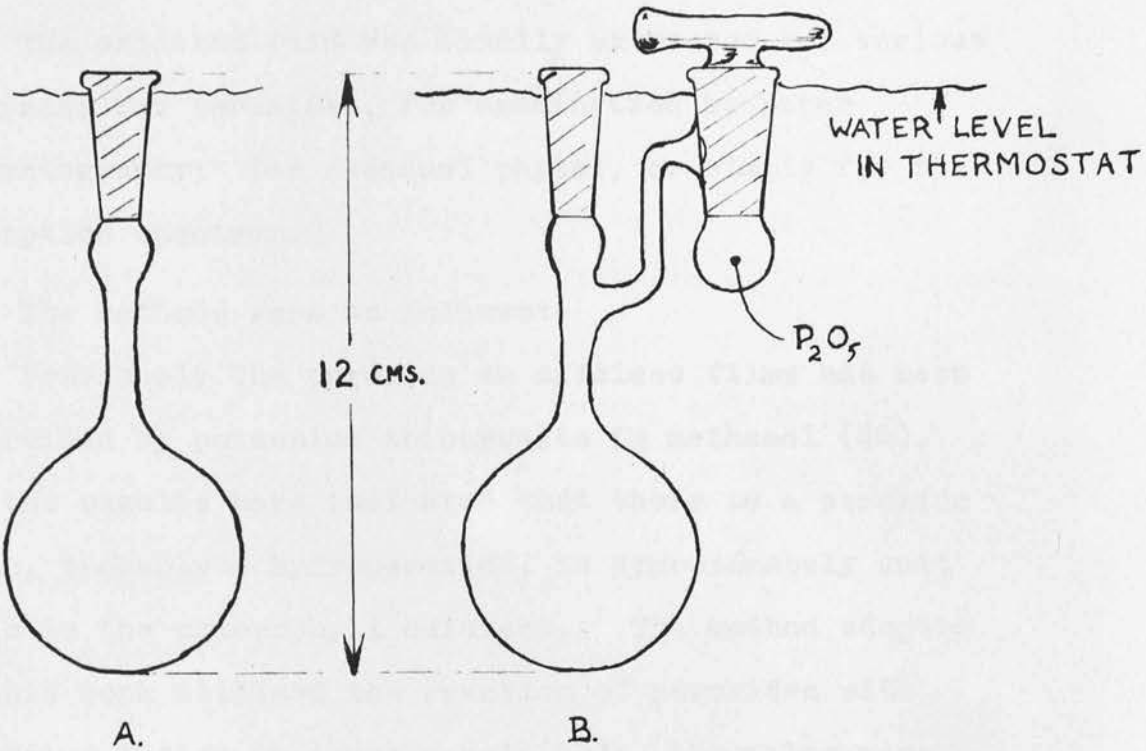
The required amount of substrate has been previously determined for the maximum rate of reaction, and was found to be 0.300 g. for a reaction vessel of 50 - 60 mls. volume. As the volumes were slightly reduced in the work to be described, and also the quantity of chlorophyll, the amount of substrate was reduced correspondingly, - to 0.200 g. In certain cases, it was found that even this was too much, as the film tended to drop into the bottom of the reaction vessel, and therefore the amount was reduced further to 0.175 g. The appropriate amount of substrate was weighed out and transferred to the reaction vessel; the required volume of pigment solution in acetone or in ether was then added by pipette, and the bulk of the solvent was removed under water pump vacuum. When the volume was small enough to form a thick paste with the substrate, the vessel was gently swirled to form a smooth film on one face of the vessel about one inch in diameter. Removal of the

solvent must proceed with great care at this stage because of the tendency for it to boil under vacuum and break up the film. The whole of the inside of the vessel was washed with a few drops of solvent to ensure that no pigment was left on the glass of the vessel, and all was transferred to the substrate; this washing was repeated three times. By making use of the property of 'creep' in chlorophyll solutions it is possible to drive the solution from the glass into the bulk of the substrate by running a drop of solvent round the edges. The film was prepared in dim artificial light, and a small light-tight box was fastened round the vessel until it was ready for illumination. It was next transferred to the reaction position and evacuated for three or four hours, and left evacuated in the dark overnight. The following morning the system was re-evacuated for an hour and rinsed out with about twenty millimetres of oxygen before admitting the required quantity for the reaction. After closing the gauge and jacket taps to isolate the system, the film was left to stand in the dark for a short time to allow the oxygen to come to thermal equilibrium (as shown by a steady pressure) before raising the shutter and commencing the reaction.

Gauge readings showing change in pressure were plotted

against time to give what will be called the 'uptake' curve; from this was derived the 'rate' curve, by plotting the change in pressure during a fixed interval of time against the total pressure change in the middle of this period of time. The length of the time interval - 5, 10 or 20 minutes - depended on the speed of the reaction. Extrapolation of the rate curve to zero rate gave an estimate of the pressure decrease for 100% reaction, and thus a ratio of the pressure decrease to the quantity of chlorophyll present can be obtained.

The reaction was stopped simply by lowering the shutter to cut off the illumination. The gaseous products were then examined in one of two ways:- a side arm on a special reaction vessel (Fig. 10 B, compared with the normal reaction vessel Fig. 10 A) containing a suitable absorbent was connected to the reaction vessel by opening the tap and any subsequent decrease in pressure was recorded, or the oxygen and gaseous reaction products were pumped out through a trap cooled in liquid oxygen, thus condensing the products for the determination of their vapour pressure curves; this method will be treated in more detail later, when a discussion of the results will show how the final details of the method came to be evolved (p. 54).



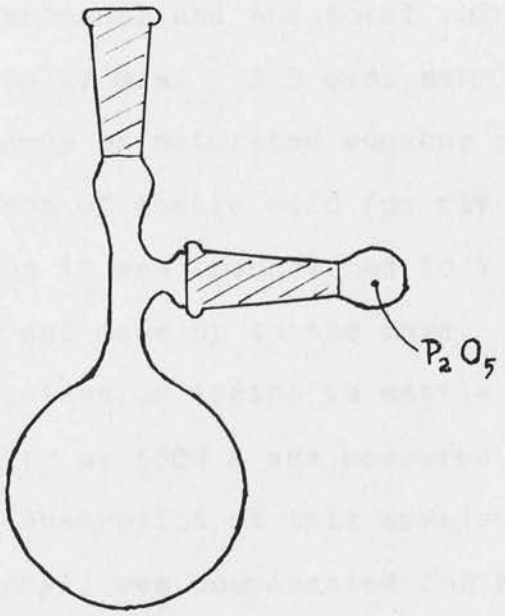


FIG. 10

The oxidised film was finally extracted for various analyses; for peroxides, for examination by paper chromatography; for residual phytol, or simply for the absorption spectrum.

The methods were as follows:-

Previously the peroxide in oxidised films has been determined by potassium thiocyanate in methanol (48), and the results have indicated that there is a peroxide group, probably a hydroperoxide, in approximately unit ratio to the chlorophyll oxidised. The method adopted in this work utilised the reaction of peroxides with potassium iodide in isopropanol (49); the films were extracted in isopropanol and the total volume of the extract made up to 20 mls. A 5 mls. sample was then heated with one drop of saturated aqueous potassium iodide and one drop of acetic acid for five minutes, and then after cooling it was transferred to a graduated flask completely and made up to the mark. After allowing any undissolved potassium iodide to settle for ten minutes, the optical density at 3800 Å was measured in 2 cms. cells in the Unicam. Absorption at this wavelength by unreacted chlorophyll was compensated for by measuring the absorption of an untreated sample and subtracting; the

intensity was also corrected for a blank on the potassium iodide solution. A standard iodine solution was prepared by dissolving 0.06 g. of resublimed iodine crystals in isopropanol and diluting five times to a concentration of about 10^{-3}N. This was then standardised against aqueous sodium thiosulphate, without using starch indicator, and the optical density measured for comparison with the unknowns.

The method adopted for the analysis for phytol is that recommended for compounds containing double bonds in 'Die Methoden der Organische Chemie' (Vol. II) (50), the decolourisation of bromine in carbon tetrachloride solution. In view of the small concentrations involved (about 10^{-5}M.), a spectro-photometric method was adopted. Carbon tetrachloride was dried for a few days over P_2O_5, and then distilled, rejecting the first 10% of the distillate. This was then used as a solvent throughout the analysis. A solution of bromine was prepared, 0.15 mls. in 50 mls., and diluted five times to about 5×10^{-6}M./ml. Five mls. of this solution was diluted to 20 mls., and then the optical density measured at the peak of the bromine absorption curve, at 4150 Å, using 2 cms. cells. To other 5 mls. samples of the bromine solution contained in 20 mls. standard

flasks were added one, two, three and four mls. of a standard solution of phytol in carbon tetrachloride, concentration about 1.5 mg./ml. = 5×10^{-6} moles / ml. Each sample was then made up to the 20 ml. mark and the decrease in the optical density measured. A straight line calibration graph was obtained, the gradient showing that one mole of phytol reacted with one mole of bromine. To apply the method, phytol films were extracted with a few mls. of carbon tetrachloride, and filtered through a No. 3 porosity sinter in the device shown in figure 11 directly into 20 mls. standard flasks; the reaction vessel and the filter were washed with more solvent, 5 mls. of bromine were pipetted in and the solution was made up to the mark. This procedure was standardised as far as possible, as the colour is not very stable. The absorption of the standard bromine solution was checked at intervals, but it was not found necessary to make any correction over the period of one or two weeks.

Paper chromatography of oxidised films (51):- Whatman No. 1 paper was used, in strips 3 x 25 cms., soaked in a solution of 90 g. of confectioners' sugar in 500 mls. of distilled water and then dried. The solvent, ½% n-propyl alcohol in n-hexane, was used without any purification.

No attempt to make the method quantitative was made, as at this stage it was only desired to make some estimate of the possibilities of the method; consequently the films were extracted in a very concentrated form with a few drops of ether and deposited onto the paper to form a dark compact spot about 5 mms. in diameter. In these cases, it was impossible to extract the film subsequently in a quantitative manner to determine the absorption spectrum. The spotting was done in dim light in a stream of nitrogen, and the papers were run in the dark in an atmosphere of nitrogen. The strips were suspended from a glass hook inserted in the rubber stopper of a gas jar, 5 x 30 cms., the ends dipping into one centimetre of solvent in the bottom of the jar. After six to eight hours, they were examined under ultra-violet light and the fluorescent spots pencilled in; this gave a sharper spot than by attempting to define the coloured spot in visible light.

Analysis for magnesium:- The following method for the analysis for magnesium was used; this was required for an estimate of the percentage of phaeophytin in degraded samples of chlorophyll. The magnesium atom is liberated by acid treatment (see p. 49) and is then

titrated with 0.0025M aqueous ethylene diamine tetra-acetic acid (disodium salt) in buffered solution using solochrome black as indicator. The endpoint was detected photometrically (using the Unicam spectrophotometer) at 6300 Å; this being the wavelength at which the absorbtion of the magnesium/indicator complex is a minimum, and that of the free dye a maximum, the magnesium being removed from the complex by E.D.T.A. (52).

Indicator: 450 mg. NH_2OH and 50 mg. solochrome black in 10 mls. of anhydrous methanol.

Buffer: 6.75 g. NH_4Cl and 57 mls. conc. NH_4OH in 100 mls. aqueous solution.

Standard: About 50 mg. of Hilger spectroscopically pure magnesium were dissolved in 10 mls. of dilute hydrochloric acid, transferred completely to a one hundred ml. graduated flask, and the solution made up to the mark with distilled water.

The cell compartment of the Unicam was fitted with a light-tight cover (Fig. 12) through which passed a small stirrer and the tip of a 5 mls. burette, both dipping into the solution contained in a Perspex cell of about 200 mls. capacity, 9 x 4 x 5 cms.

A known volume of standard magnesium solution (or the acid treated chlorophyll sample) sufficient to give a

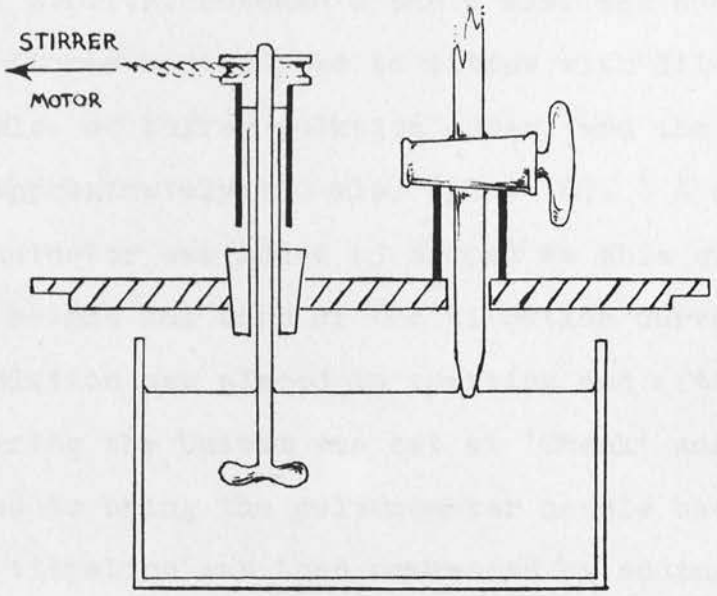

STIRRER
MOTOR

FIG. 12

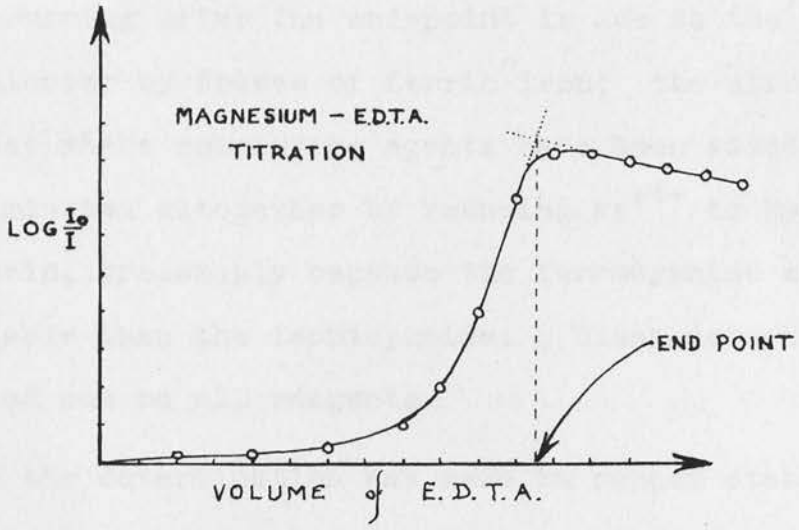

MAGNESIUM – E.D.T.A.
TITRATION
LOG $\frac{I_0}{I}$
END POINT
VOLUME of E.D.T.A.

FIG. 13

titration of E.D.T.A. between 2 and 4 mls. was added to the cell. It was neutralised to litmus with dilute ammonia, 2 mls. of buffer solution added, and the volume made up to approximately 100 mls. (pH = 10). A constant amount of indicator was added (3 drops) as this quantity decides the height and form of the titration curve. The cell with solution was placed in position and after a few minutes stirring the Unicam was set at 'Check' and the slit adjusted to bring the galvonometer needle back to zero. The titration was then commenced by adding E.D.T.A. in small amounts and reading the corresponding optical densities every minute. By plotting the volume of E.D.T.A. added against the optical density, a graph of the form shown in Fig. 13 is obtained. The decrease in the optical density occurring after the end-point is due to the bleaching of the indicator by traces of ferric iron; the effect is not so great where complexing agents have been added, and may be eliminated altogether by reducing Fe^{+++} to Fe^{++} with ascorbic acid, presumably because the ferrocyanide complex is more stable than the ferricyanide. Blank determinations were carried out on all reagents.

Where the determination was made in copper stabilised chlorophyll, the copper in the solution had to be complexed with cyanide. Sufficient ammonium tartrate to form a ten-

fold excess (molar) over the copper present was added in a few mls. of distilled water, the solution was neutralised to litmus with ammonium hydroxide, two mls. of buffer were added, and then a ten-fold molar excess of potassium cyanide. The mixture was finally warmed to 70°C. on a hot-plate for a few minutes, cooled, and transferred to the titration cell for analysis.

Standardisation results:

Titration	Magnesium (mls.)	E.D.T.A. (mls.)	Average	Normality of EDTA x 10^3
Reagent blank	-	0.09 (0) 0.08 (4)	0.08 (7)	-
Standardisation	4.00	3.27 (3) 3.27 (0) 3.22 (8)		2.40 (7) 2.40 (9) 2.43 (5) 2.41 (7)
Copper complexing reagent blank	-	0.18 (6) 0.17 (8)	0.18 (2)	-
Standardisation with complexing reagents -	4.00	3.28 (0) 3.21 (4)		2.40 (2) 2.45 (2) 2.42 (2)

The normality of the standard magnesium was 1.96 (9) x 10^{-3}. Titrations to determine normality are corrected for their respective blanks. The average normality is therefore $\underline{2.42 \times 10^{-3}}$, the standard deviation being about ½%. There appears to be no difference in the titres with and without

the presence of the copper complexing reagents after correction for the blanks.

Determination of Copper : This was made with disodium diethyl dithiocarbamate, by comparing the intensities of colour developed in standards and unknown on the Unicam at 4600 Å (53).

Standard copper: prepared from $CuSO_4.7H_2O$,
concentration = 1 mg. Cu / ml.

Carbamate solution: 0.1% aqueous.

Approximately 1 g. of the commercial paste was dried for a few days over P_2O_5 to constant weight and then ashed. The ash was dissolved in 1 : 1 nitric acid, the solution made up to 50 mls., and finally diluted ten times.

25 mls. of dilute ammonium hydroxide and 5 mls. of carbamate solution were added by pipette to 5 mls. samples of copper solution (standard or unknown). The colour developed was measured immediately on the Unicam in 1 cm. cells, and by Beers' law, the intensities developed in the two solutions are directly proportional to the concentration of copper in each.

EXPERIMENTAL RESULTS.

It has been previously established that illumination of partially substituted copper chlorophyll in the presence of oxygen results in a pressure decrease in unit ratio to the chlorophyll present. Attempts to extend this result to samples of chlorophyll extracted from spinach showed that although it could be obtained, it was not so reproducible, and tended to become greater than one (54). Now replacement of 1 in 10 magnesium atoms with copper, as revealed by analysis, results in complete quenching of fluorescence, the phase test becomes negative, and solutions become more resistant to bleaching by light. The overall effect may therefore be regarded as stabilisation, (copper being a better co-ordinating atom than magnesium) and the lack of reproducibility in natural chlorophylls and increased uptakes to instability and consequent degradation. The commonest form of degradation in chlorophyll is the loss of magnesium with the formation of phaeophytin; this occurs on drying, standing in the dark, and in the absence of oxygen. It has been shown that phaeophytin, oxidised under the same conditions, gives an extrapolated pressure decrease of three. The degradation can be detected spectroscopically, but not with any degree of accuracy in the present conditions. It was therefore decided to develop a method for the analysis for magnesium, to attempt

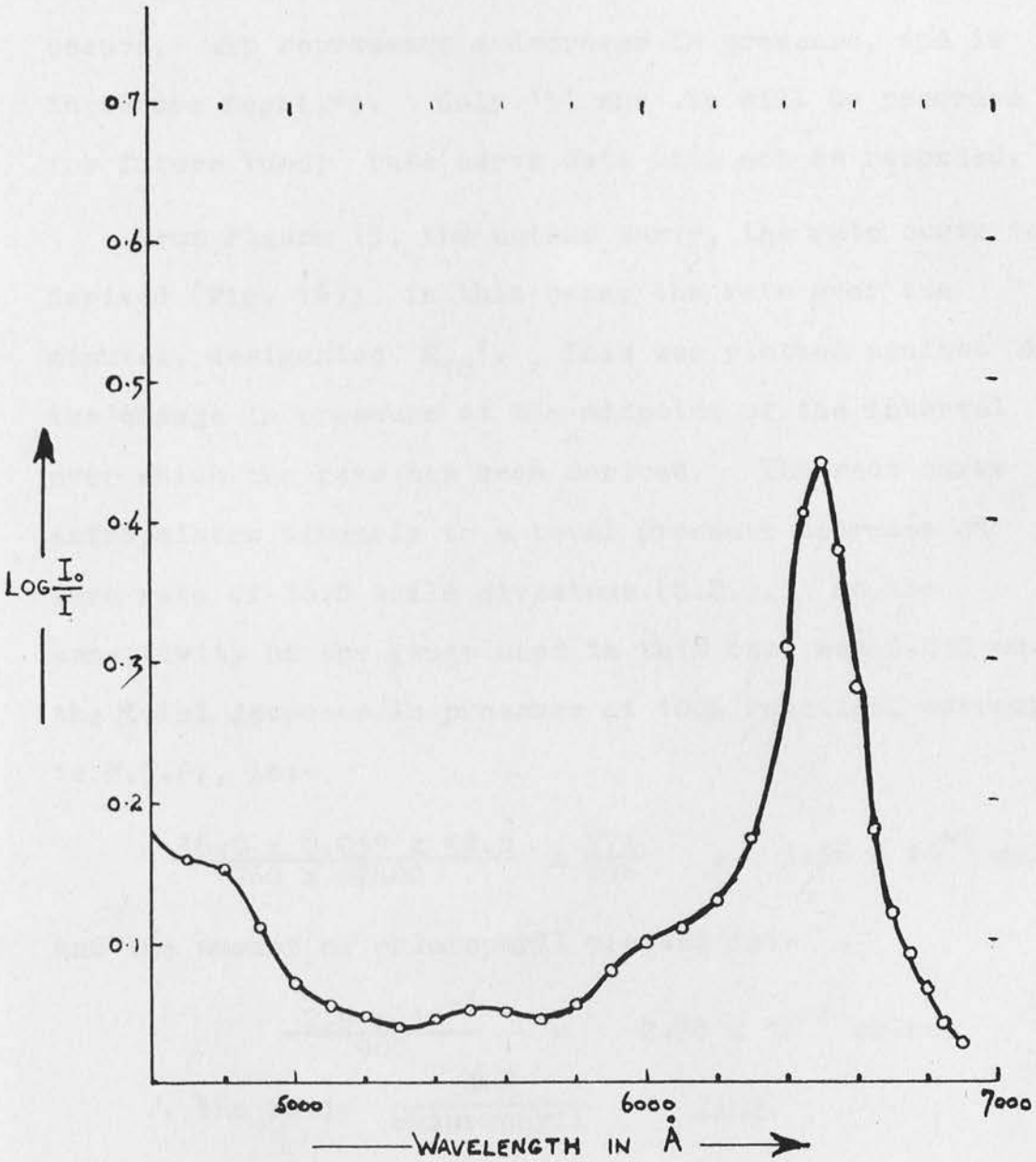
to correlate increased uptakes with loss of magnesium, i.e. with increase in the amount of phaeophytin present.

Run 13. Re-examination of a sample of copper stabilised chlorophyll (McFarlane's, Edinburgh, 'ZZ') dated 1951, oxidising up to 1953 in a unit molar ratio; re-examined 1956. The absorption spectrum of a purified sample (method - p.27) is shown in Figure 14.

A film was prepared from 0.200 g. of thallous bromide, and 2.00 mls. of a bonemeal-purified solution of chlorophyll in acetone, concentration 1.25 mg./ml. The film, in a reaction vessel of 52.8 mls. volume, (when attached to the gauge) was attached to the Bourdon gauge and evacuated to 10^{-4} mm. for $2\frac{1}{2}$ hours, and left in the dark at this pressure overnight. The system was then re-evacuated to 10^{-4} mm. for 50 mins., and 100 mm. of oxygen admitted directly to the gauge and jacket. The gauge and jacket taps (T_2 and T_6, Fig.6) were closed, and the pointer read at five minutes intervals for twenty minutes, (or longer if a steady reading had not by that time been reached). The shutter was raised and the illumination commenced; the gauge was then read every five minutes, or ten minutes when the reaction had become slower, and the results were tabulated as under (table 1.), and plotted as on Figure 15. The column marked 't' represents the time in minutes since

FIG. 14

ABSORPTION SPECTRUM of COPPER CHLOROPHYLL.



the start of the reaction, and that marked 'G', the gauge reading at that time. The third column, Δp, represents the change in pressure, measured in scale divisions from the beginning of the reaction. As a decrease in pressure occurs, Δp represents a decrease in pressure, and is therefore negative. Only 't' and Δp will be recorded for future runs; rate curve data will not be recorded.

From Figure 15, the uptake curve, the rate curve is derived (Fig. 16); in this case, the rate over ten minutes, designated 'R_{10}'. This was plotted against Δp, the change in pressure at the midpoint of the interval over which the rate has been derived. The rate curve extrapolates linearly to a total pressure decrease at zero rate of 36.0 scale divisions (S.D.). As the sensitivity of the gauge used in this case was 0.035 mm./S.D., the total decrease in pressure at 100% reaction, corrected to N.T.P., is:-

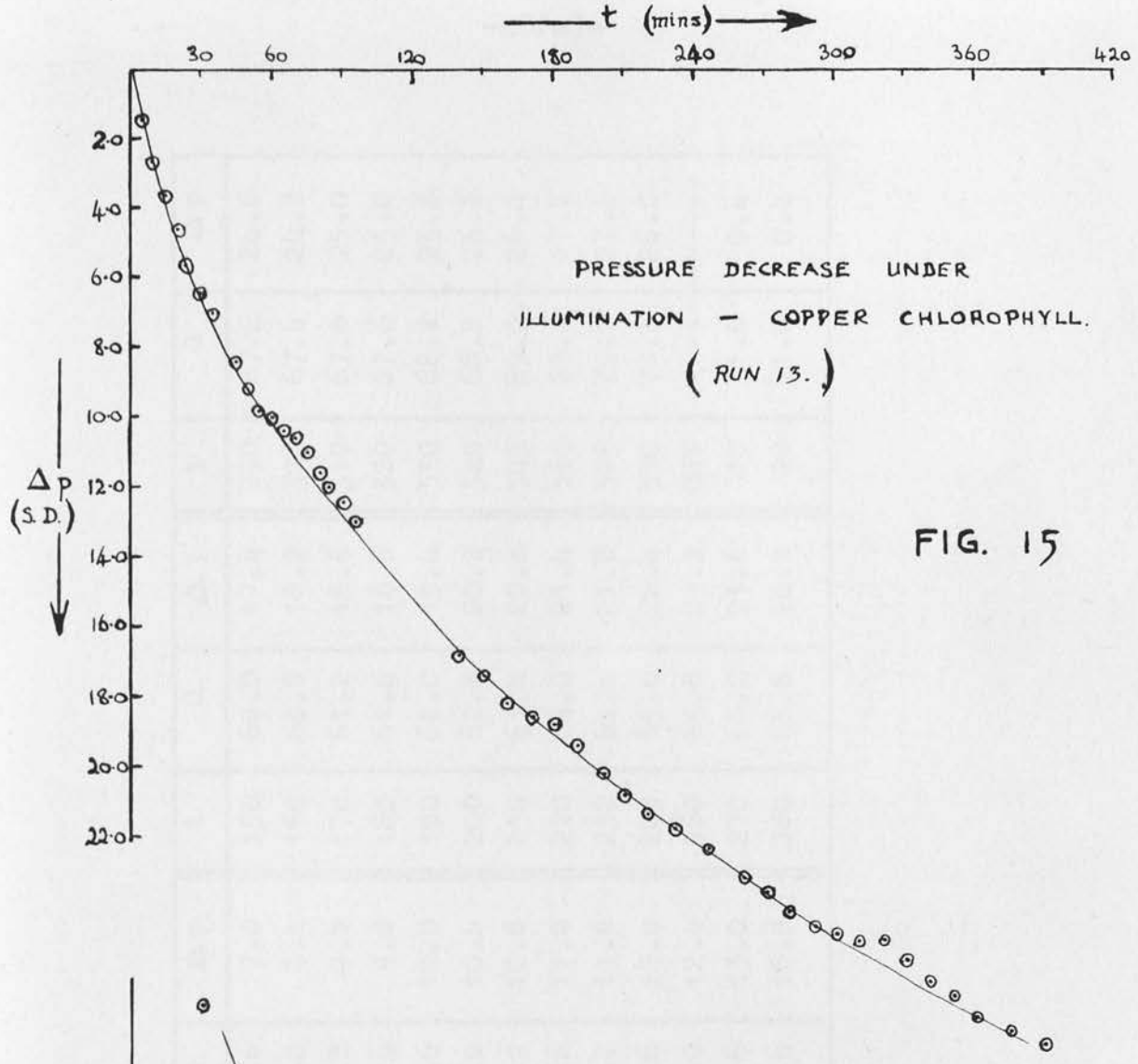
$$\frac{36.0 \times 0.035 \times 52.8}{760 \times 22400} \times \frac{273}{298} = 3.58 \times 10^{-6} \text{ moles,}$$

and the amount of chlorophyll present is:-

$$\frac{2.5 \times 10^{-3}}{900} = 2.78 \times 10^{-6} \text{ moles.}$$

$$\therefore \text{ the ratio } \frac{\Delta p}{\text{chlorophyll}} = \underline{1.29}.$$

This ratio will be referred to in future as the



PRESSURE DECREASE UNDER ILLUMINATION – COPPER CHLOROPHYLL.
(RUN 13.)

FIG. 15

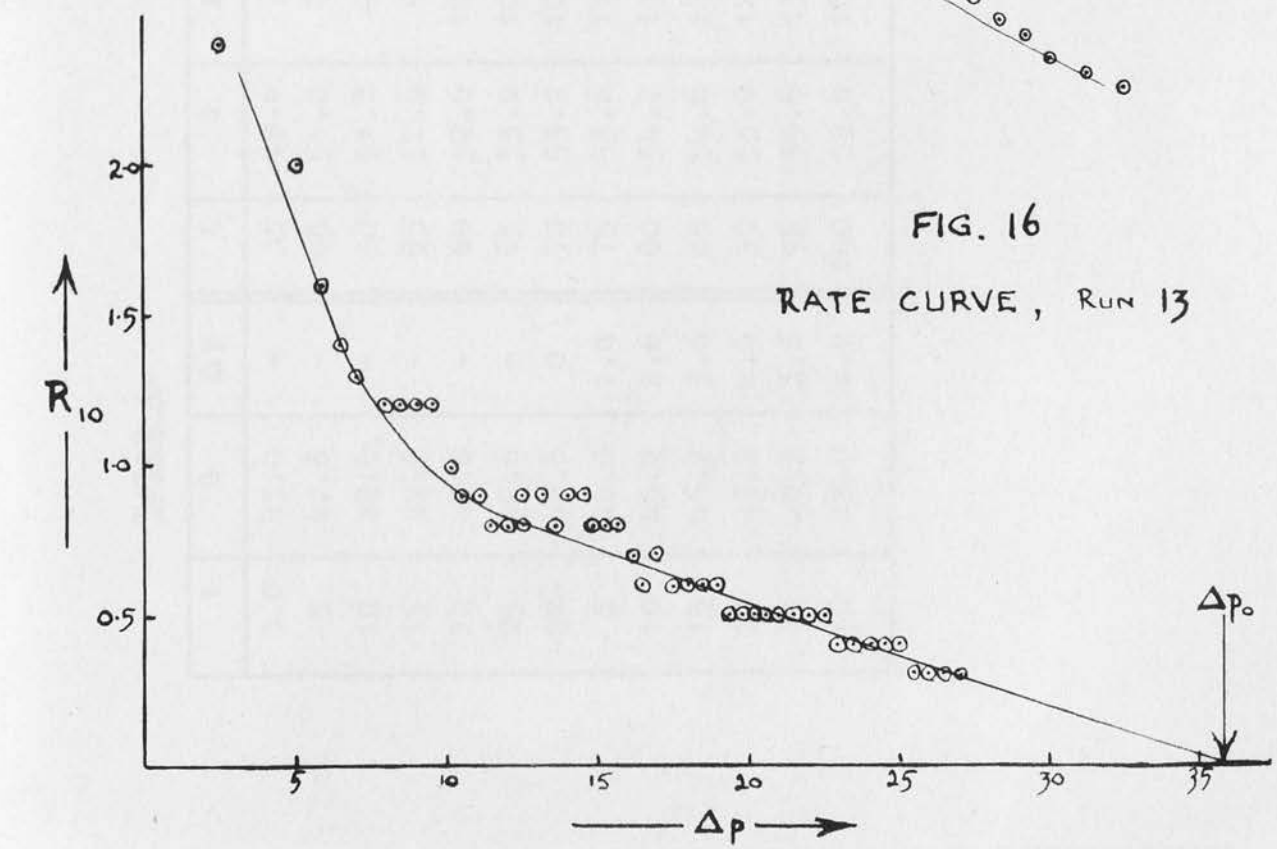


FIG. 16

RATE CURVE, RUN 13

TABLE 1.

t	G	Δp	t	G	Δp	t	G	Δp	t	G	Δp
0.0	43.0	-	35	49.6	7.0	150	60.0	17.4	290	67.2	24.6
5	42.8	-	45	51.0	8.4	160	60.8	18.2	300	67.4	24.8
10	42.6	-	50	51.8	9.2	170	61.2	18.6	310	67.6	25.0
15	42.6	-	55	52.4	9.8	180	61.4	18.8	320	67.6	25.0
20	42.6	-	60	52.6	10.0	190	62.0	19.4	330	68.2	25.6
25	42.6	-	65	53.0	10.4	200	62.8	20.2	340	68.8	26.2
-ON-	42.6	0	70	53.2	10.6	210	63.4	20.8	345	69.0	26.4
5	44.0	1.4	75	53.6	11.0	220	64.0	21.4	360	69.8	27.2
10	45.2	2.6	80	54.2	11.6	230	64.4	21.8	375	70.2	27.6
15	46.2	3.6	85	54.6	12.0	245	65.0	22.4	390	70.6	28.0
20	47.2	4.6	90	55.0	12.4	260	65.8	23.2	OFF	----------	----------
25	48.2	5.6	95	55.6	13.0	270	66.2	23.6	10	71.0	0.4
30	49.0	6.4	140	59.4	16.8	280	66.8	24.2	20	71.0	0.4

TABLE 2.

R_{10}	2.6	2.4	2.0	1.6	1.4	1.3	1.2	1.2	1.2	1.2	1.0	0.9
Δp	1.5	2.5	5.0	5.8	6.6	7.0	8.0	8.4	9.0	9.6	10.2	10.6
R_{10}	0.9	0.8	0.8	0.8	0.9	0.9	0.8	0.9	0.9	0.8	0.8	0.8
Δp	11.1	11.6	12.0	12.4	12.6	13.2	13.5	14.0	14.5	14.8	15.3	15.6
R_{10}	0.7	0.6	0.7	0.6	0.6	0.6	0.6	0.5	0.5	0.5	0.5	0.5
Δp	16.2	16.6	17.0	17.5	17.9	18.5	19.0	19.4	19.6	20.0	20.6	21.0
R_{10}	0.5	0.5	0.5	0.4	0.4	0.4	0.4	0.4	0.3	0.3	0.3	
Δp	21.4	22.0	22.5	23.0	23.5	24.0	24.5	25.0	25.5	26.0	26.5	

"pressure ratio". Thus from the original unit value, the ratio has changed little in five years, but the indication is that any degradation with time will take the same course in copper chlorophyll as it does in natural chlorophyll, i.e. it will tend to increase; it is probable that the cause is the same in both cases.

Analysis for magnesium:-

Preliminary experiments (55) showed that in spite of the reputed sensitivity of the magnesium atom in chlorophyll to acids, the treatment necessary to liberate the magnesium in a titratable form would be source of difficulty in this determination, the actual titration being a fairly standard method. The preliminary experiments consisted of shaking an acetone solution of chlorophyll with various amounts of acid:- (a) about 6 mg. of chlorophyll were shaken overnight with twice the molar equivalent of HCl in 10 mls. of acetone/water solution, titration revealed no magnesium in the extract; (b) 6 mg. chlorophyll were shaken overnight with ten times the molar equivalent of HCl, when only 0.5% magnesium was revealed; and (c) 2 mg. of chlorophyll was shaken overnight with two drops of concentrated HNO_3 and two drops of concentrated HCl in 5 mls. of acetone, this time 1.6% of magnesium was revealed. Thus it appeared necessary to do some preliminary work on the acid treatment

required to liberate the magnesium; for convenience on copper chlorophyll. The results are given below:-

TABLE 3 : acid treatment of chlorophyll.

No.	Treatment	Acid	%age Mg.
1.	Evaporated down	HNO_3	0.09
2.	Evaporated down twice	HNO_3	0.38
3.	Evaporated once	HNO_3 + H_2SO_4	No titration
4.	Shaken with excess of copper sulphate	---	0.03
5.	Shaken overnight with	aqua regia	2.06
6.	Evaporated down	aqua regia	0.17
7.	Standing 16 hours	aqua regia	2.26
8.	Shaken overnight, then evaporated down	aqua regia	2.28

The maximum amount of magnesium is revealed after shaking or even just standing overnight in aqua regia; it does not even appear to be necessary to evaporate down the volume of acid by gentle warming on a hot-plate. If the evaporation process is taken too far, a black crusty deposit forms; this occurred in experiment 8, but does not seem to have affected the result. No titration was obtained in experiment 3; the indicator appeared to fade rapidly, possibly because of the presence of sulphuric acid.

Practically no magnesium was revealed in experiment 4; it was later found that simple shaking with copper sulphate did not quench the fluorescence of a sample of spinach chlorophyll and therefore probably did not replace the magnesium atom.

Approximately 1 ml. of acid was used in each experiment, to decompose 2 mg. of chlorophyll. On the basis of these results, the technique adopted was to stand the sample overnight in 1 ml. of acid, and then to reduce the volume next morning by gentle warming on a hot-plate almost to dryness, before neutralising to litmus with conc. NH_4OH for the titration.

Now replacement of some magnesium by 0.7% copper by weight should leave (2.70 - 0.27) = 2.43% magnesium in the organic material; a pressure ratio of 1.3 requires, (if the increase is due to the presence of phaeophytin) (2.70 - 0.41) = 2.29% magnesium; the above result of 2.28% is therefore significant. In the case of spinach chlorophyll, similar results were obtained. The absorption spectrum changed over a period of three months to show the phaeophytin band at 5050 Å, and the pressure ratio became 1.70. The absorption spectra, and the corresponding pressure ratios are shown in figures 17 and 18. Now a ratio of 1.70 will require 1.62% of magnesium in the molecule

FIG. 17

ABSORPTION SPECTRUM of PURE CHLOROPHYLL.

l = 1 cm., c = 0.0138 g. / l.

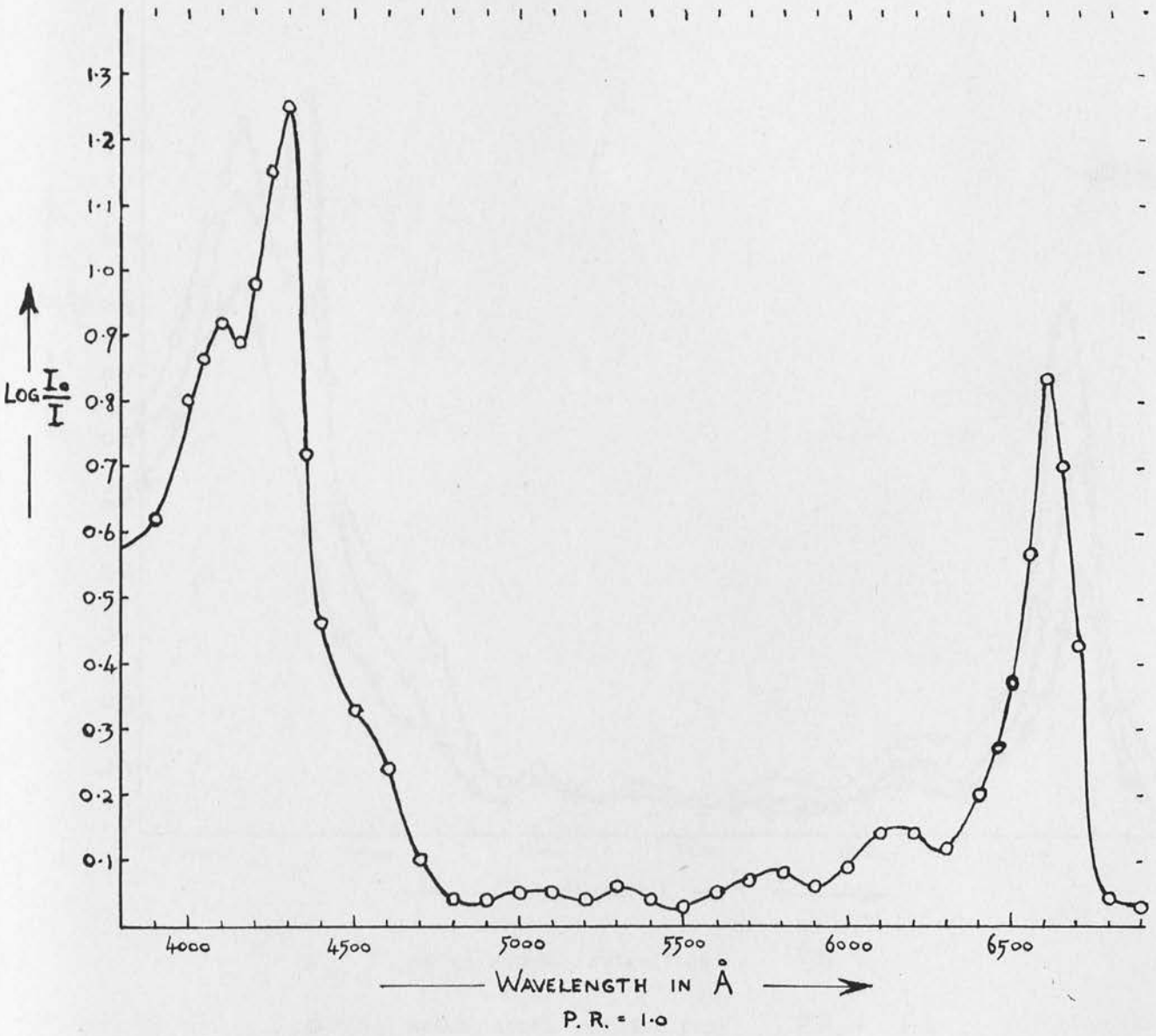
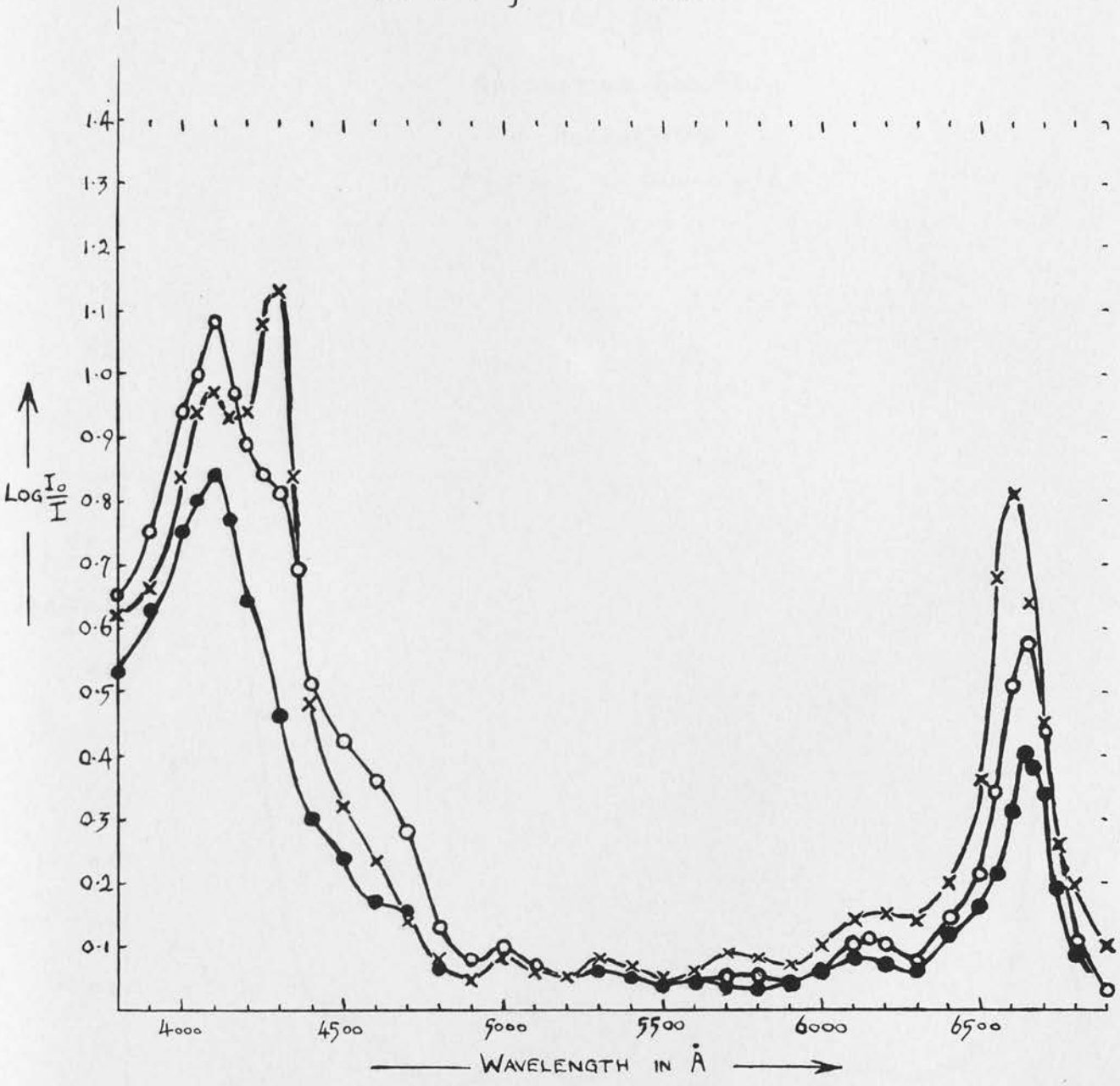


FIG. 18

ABSORPTION SPECTRA of DEGRADED SAMPLES of CHLOROPHYLL.



× 7 WEEKS AFTER EXTRACTION P.R. ≒ 1

○ 11 WEEKS AFTER EXTRACTION P.R. ≒ 1·4

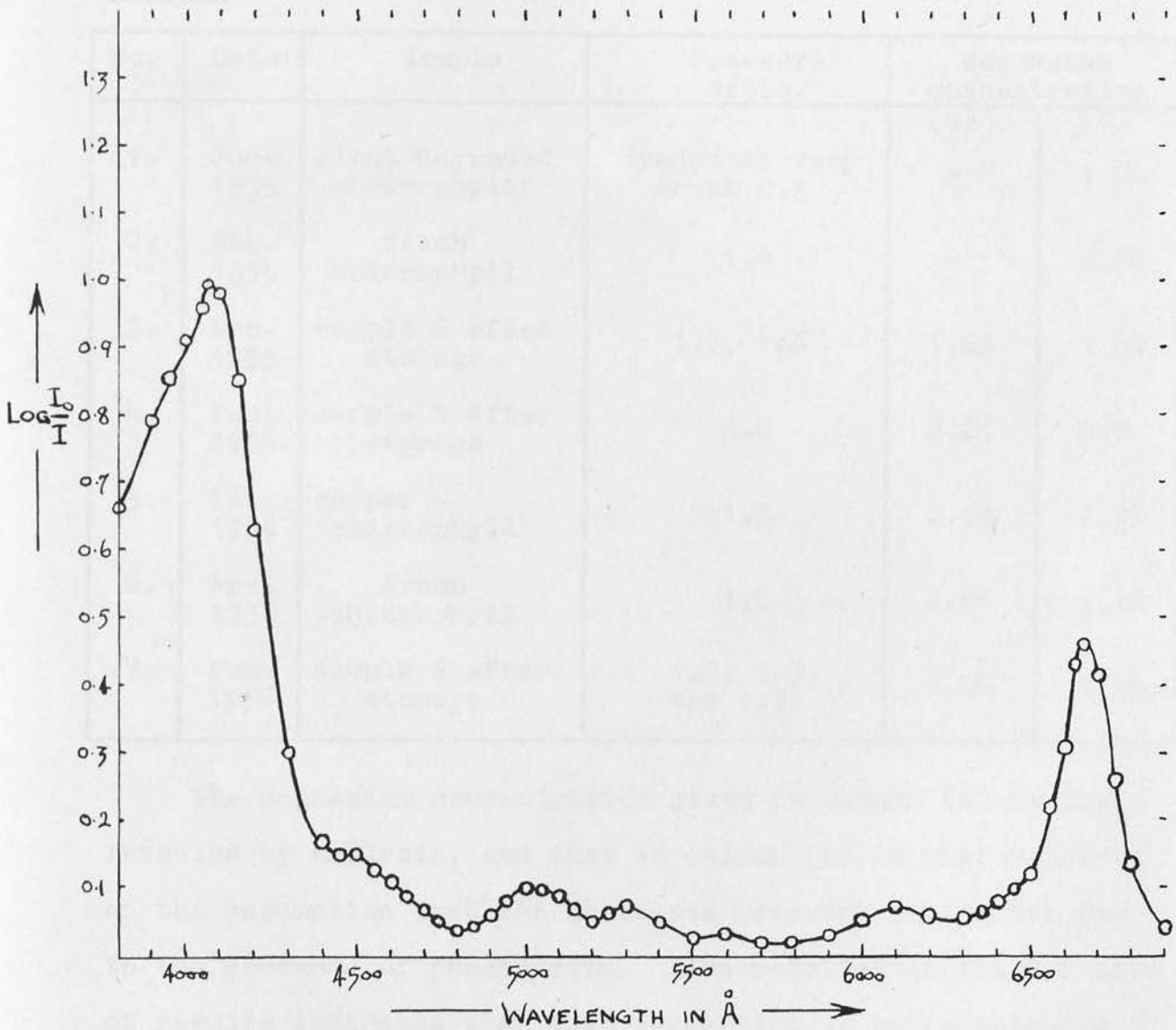
● 14 WEEKS AFTER EXTRACTION P.R. ≒ 1·7

FIG. 19

ABSORPTION SPECTRUM

of PHAEOPHYTIN

$l = 1$ cm., $c = 0.0140$ g/l.



if the increase is due to phaeophytin, (as the spectra seem to suggest), the figure obtained by analysis is 1.60%. Degraded samples of chlorophyll were passed down a small sugar column before analysis to remove any free magnesium.

TABLE 4. Magnesium analysis:

No.	Date	Sample	Pressure Ratio.	Magnesium concentration	
				(a)	(b)
1.	June 1955	plant degraded chlorophyll	tended to vary about 2.3	-	1.0%
2.	Aug. 1955	fresh chlorophyll	1.0	-	2.7%
3.	Dec. 1955	sample 2 after storage	1.7, 1.6	1.6%	1.6%
4.	Feb. 1956	sample 1 after storage	3.0	0.2%	0.0
5.	Feb. 1956	copper - chlorophyll	1.3	2.3%	2.3%
6.	Apr. 1956	fresh chlorophyll	1.0	2.6%	2.7%
7.	June 1956	sample 6 after storage	1.5, 1.3, and 1.7.	2.4%	2.3%

The magnesium concentration given in column (a) is that revealed by analysis, and that in column (b) is that required on the assumption that the increased pressure ratios are due to the presence of phaeophytin. The parallel in the two sets of results indicates that this assumption is satisfactorily valid.

It is seen from the table that once a sample starts to degrade and give pressure ratios greater than one, the ratio is no longer reproducible, even at the higher value. The reason for this is not quite clear, but table 5 below shows the pigment becomes very much more sensitive to the handling it receives:-

TABLE 5 :

Run	Sample of:	Substrate	Standing Evacuated	Pressure Ratio
2.	Chlorophyll	Glass	2 days	2.62
3.	Chlorophyll	TlBr	2 days	2.37
5.	Chlorophyll	TlBr	16 hours	2.23
6.	Chlorophyll	Glass	16 hours	2.40
7.	Phaeophytin	Glass	16 hours	3.22
8.	Chlorophyll + H_3PO_4.	Glass	16 hours	3.08

The sample used in these runs was extracted from spinach 24 hours after cutting; this may be the reason for the high pressure ratios. Subsequently the extraction was made as soon as possible after cutting. The phaeophytin used in run 7 was prepared from this sample of chlorophyll by the method described on page 33, and although the ratio is higher than the previous results of three, it was taken to show that the high chlorophyll ratios were probably due

to the same effect that caused other ratios with natural chlorophyll to be high. Further work on phaeophytin showed that the pressure ratio was frequently slightly greater than three (p. 66).

Run 8 was performed on a film of equimolar amounts of chlorophyll and phosphoric acid; this apparently has the same effect as depositing phaeophytin directly on the substrate instead of allowing it to form during the deposition, as here. There was no detectable difference in form for the rates curves of these two samples; the rate curve for phaeophytin is discussed in that section.

Investigation of the gaseous reaction products by means of vapour pressure curves:-

If the oxidation of chlorophyll is carried out in the presence of various absorbents, in a reaction vessel as in Fig. 10B or 10C, then increased pressure ratios are obtained. This indicates that the decrease in pressure is not due simply to the uptake of one molecule of oxygen, but is the resultant of the uptake of 'n' molecules of oxygen followed by the release of 'n-1' molecules of some gas taken up by the absorbents. As absorbents, both P_2O_5 and soda-lime have been used previously (40), but with neither of these were reproducible results obtained. Values tended towards five or six, and were greater with soda-lime and P_2O_5 than with

P_2O_5 alone.

It was not considered practicable to use soda-lime and P_2O_5 together in the same system, as for maximum efficiency, soda-lime must contain about 13% water (56). This may account for some of the previous results.

Qualitatively, the gaseous products of the reaction have been shown to be CO_2, H_2O, and an intermediate fraction which could be acetone, or any other organic liquid with a very similar vapour pressure curve, in the approximate proportions 4 : 90 : 6. To make the analysis more quantitative, and in order to investigate the apparent increasing complexity of the reaction, the following method was adopted:-

After every run, the oxygen and products were pumped out through a cleaned and evacuated trap, cooled in liquid oxygen, at 'A' in Figure 6. Any condensed products were distilled for 15 minutes through the evacuated tubing isolated by taps 1, 2, 4 and 5, into a small vessel (5 - 10 mls. volume) attached by an A10 ground glass joint below a tap 4 to the main vacuum line. The reaction vessel with the exhausted film was then replaced by a smaller clean vessel which was evacuated to about 10^{-3} mm., together with the frozen products. Finally the refrigerant around the products was replaced by a vacuum flask cooled to the temperature of liquid oxygen. This was allowed to warm

up slowly to room temperature and the products were expanded into the gauge. By measuring the temperature of the thermos flask with a pentane thermometer, or of a special product vessel with a thermocouple inserted, and plotting the temperature against the increase in pressure recorded on the gauge, a graph of the form shown in Figure 20 was obtained. In this particular instance, the experimental curve is plotted against points derived from 'International Critical Tables' for the vapour pressure curves of each suspected component; it is seen that the first can be identified with carbon dioxide, the third with water, and the middle one with two or three organic compounds. Normally, to determine the quantity of each component present, it was found sufficient to plot pressure against some function of temperature, or even against time.

The irreproducibility of the first results (table 6) showed that this simple procedure was not sufficient to give accurate quantitative information about the products. The following refinements were therefore made to the method: the oxygen and products were pumped out to just below one millimetre of mercury through a cooled trap in the usual way, the products were distilled to a vessel and stored for subsequent analysis; the remaining gas was pumped out by the oil pump to 10^{-3}mm. and stored similarly; and finally

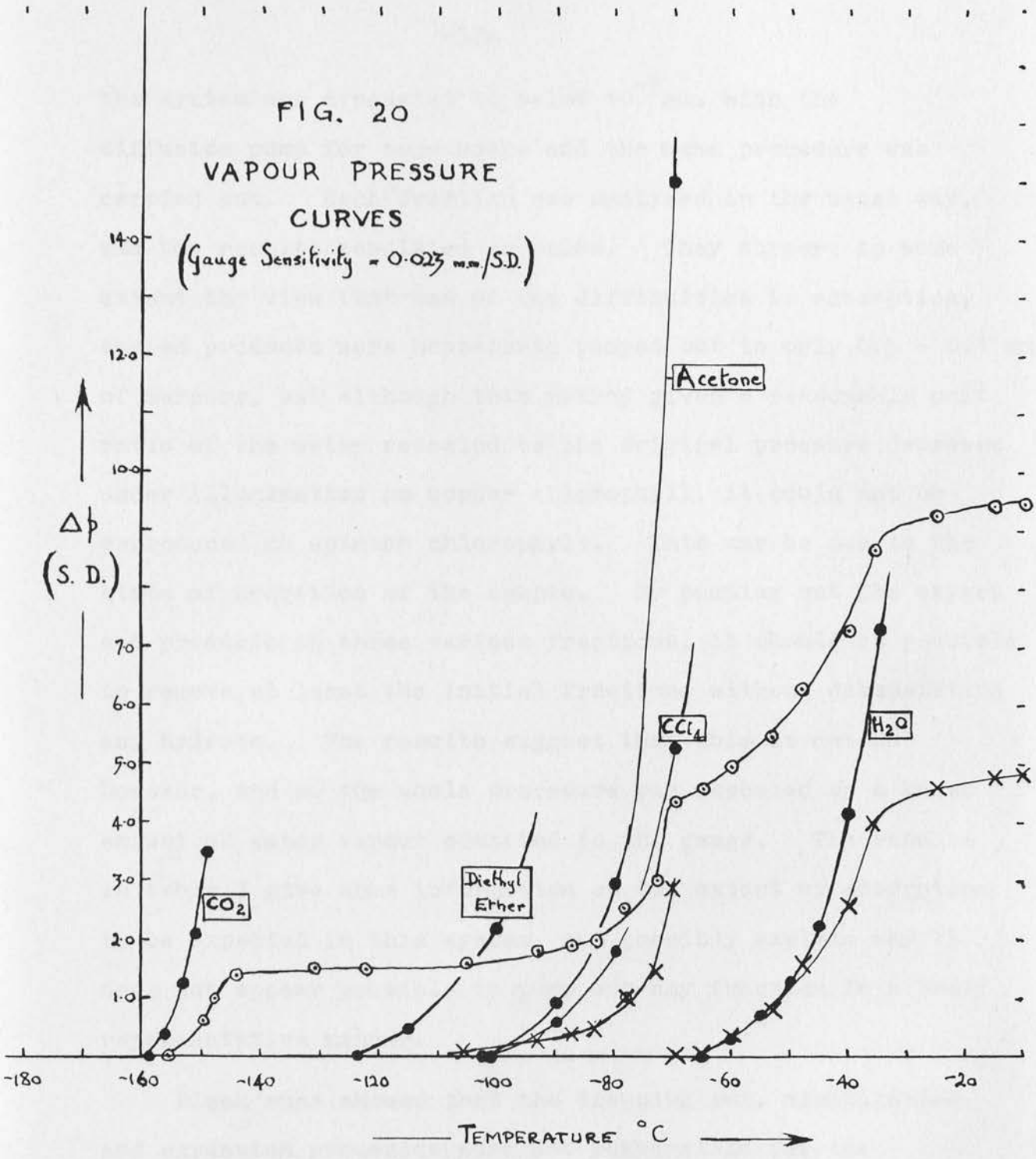


FIG. 20
VAPOUR PRESSURE CURVES
(Gauge Sensitivity = 0·023 mm/S.D.)
Δp (S. D.)
14·0
12·0
10·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
Acetone
CCl4
H2O
Diethyl Ether
CO2
-180
-160
-140
-120
-100
-80
-60
-40
-20
TEMPERATURE °C
• Points from I.C.T.
o Experimental Points
× Experimental points, each fraction corrected to Zero.

the system was evacuated to below 10^{-5}mm. with the diffusion pump for some hours and the same procedure was carried out. Each fraction was analysed in the usual way, and the results tabulated as below. They support to some extent the view that one of the difficulties is adsorption, and so products were henceforth pumped out to only 0.5 - 0.1 mm. of mercury, but although this method gives a reasonable unit ratio of the water revealed to the original pressure decrease under illumination on copper chlorophyll, it could not be reproduced on spinach chlorophyll. This may be due to the state of hydration of the sample. By pumping out the oxygen and products in three various fractions, it should be possible to remove at least the initial fractions without dissociating any hydrate. The results suggest that this is not so however, and so the whole procedure was repeated on a known amount of water vapour admitted to the gauge. The results in table 7 give some information on the extent of adsorption to be expected in this system, and possibly explain why it does not appear possible to pump out any fraction in a truly representative manner.

Blank runs showed that the trapping out, distillation, and expansion processes were not responsible for the discrepancies. It should be remembered that chlorophyll is hydrated to an unknown degree, and, if it is possible for

some of the water of hydration to be released after reaction and not before, this may be responsible for some of the high values obtained. All the runs below were done on the one sample of spinach chlorophyll, oxidising in unit ratio, and showing the typical acceleration on re-illuminating in oxygen after evacuation. Between 1.6 and 2.3 mg. of pigment were used in each run, depending on the concentration of the solution obtained from the columns after each purification.

A typical rate curve for such a sample of chlorophyll is shown in Figure 21, and the absorption spectrum is given in Figure 17.

It should be noted that because of the difference in the reaction volume and the total volume into which the products are expanded, a correction is necessary when comparing the decrease in pressure under illumination and the pressure of each product component revealed.

The results do show, however, that though the amount of water revealed is often greater than the pressure decrease under illumination, it exceeds it by a factor of five in one case only (discounting final fractions). The pressure ratios of nearly five found in runs performed in the presence of P_2O_5 are therefore not dependable, and it would appear that the value of (n - 1), the number of water molecules released, is more likely to be 1 or 2.

TABLE 6: The determination of water by vapour pressure measurements

All runs were made on a thallous bromide substrate, except where indicated; and all quantities are expressed in scale divisions.

Where the products have been pumped out in more than one fraction, the amount of water shown below is the amount revealed in that fraction corrected to the total, i.e. in run 16, the amount of water revealed in the first 50 mm. fraction was 4.1 S.D., thus there should be on this basis, a total of 8.2 S.D. of water altogether.

Run	Sample of:	Pressure decrease	Total amount of water present; (each fraction corrected to the total).	Products in three fractions:-				
				100 to 1mm.	1mm. to 10^{-3}mm.	10^{-3}mm. to 10^{-5}mm.		
10	Chlorophyll	14.2	One fraction only, to 10^{-4}mm., H_2O = 27.0					
11	Chlorophyll	15.6	One fraction only, to 10^{-4}mm., H_2O = 47.0					
12	Chlorophyll on glass	13.5	One fraction only, to 10^{-4}mm., H_2O = 9.6					
13	Copper chlorophyll	28.0		26.4	0	7.0		
14	Chlorophyll	5.6		21.0	Not examined.	3.2, obtained before reaction		
15	Chlorophyll	8.0		21.0	Not examined.	2.8, obtained before reaction		
				100 mm. to 50 mm.		50 mm. to 1 mm.		
16	Chlorophyll	4.6		8.2		11.4		
				100mm. to 75mm.	75mm. to 50mm.	50mm. to 1mm.		
17	Chlorophyll	9.6		11.2	7.8	64.0		
18	Chlorophyll	9.6		20.0	10.6	36.8		
19	Chlorophyll	10.2		40.6	Not examined.	78.2		
20	Chlorophyll	11.8		17.5	12.0	21.6		

TABLE 7: absorption of water vapour in a reaction vessel containing an oxidised film of chlorophyll.

Again all quantities are expressed in scale divisions; 10 minutes were allowed for adsorption to be completed, as only 5 - 10 % more water was taken up in a further 15 minutes.

No.	Amount admitted	Decrease (10mins.)	Second admittance	Decrease (10mins.)	Total admitted	Free water	Free/Total %	Adsorbed%
1	30.2	8.2=27%	12.2	None	42.4	27.2	64.2	35.8
2	40.0	11.2=28%	14.4	2.6	54.4	41.0	75.0	25.0
3	20.6	1.8=8%	14.8	1.0	35.4	32.6	92.4	7.6

'Free' water is that revealed by freezing and expanding from and into the gauge, readings being taken after 10 minutes. The 'free' water in experiment No. 3 was increased to 40.0 scale divisions after 35 minutes freezing in liquid oxygen; the ratio of free water to total admitted is now 114%. Experiment 3 was carried out in a clean reaction vessel, evacuated to 5×10^{-4} mm. before commencing the determinations.

Determination of the amount of water produced in the reaction by absorption in phosphorus pentoxide.

This was investigated as an alternative method to the vapour pressure method. The oxidations were carried out in a special reaction vessel (Fig. 10B) having a side-arm which could be closed

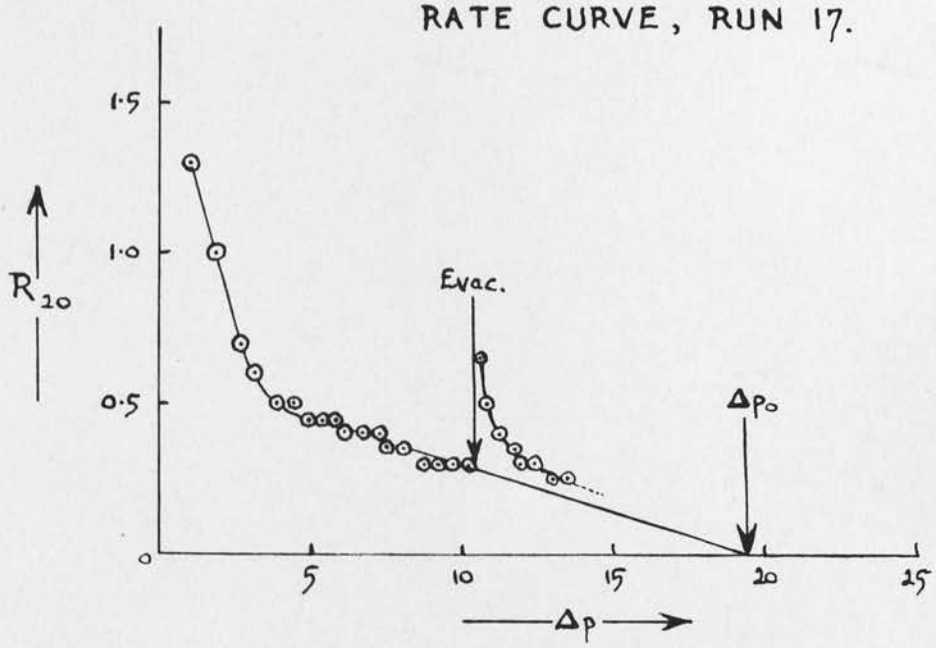
off from the main reaction space by a tap. Initially, this vessel was designed so that the tap was under water, but although a ground glass joint under water appears to be perfectly satisfactory, the tap was a continual source of leaks and a potential danger to the gauge.

The method of analysis was as follows: after the required pressure decrease had occurred, the illumination was stopped, the tap isolating the absorbent was opened, and the resulting dark pressure decrease was recorded until the process was complete, as shown by a steady pressure; this usually took one or two hours. The side-arm was given the same pre-reaction treatment as the film, as described on p.36, except that overnight the tap was turned off, both to prevent contamination of the absorbent by vapours given off by the film (really only necessary in the case of acetone-deposited films), and to prevent excessive drying of the film, as some reactions on TlBr have been shown to stop short of 100% if the films are too dry. After admitting oxygen to the reaction vessel, the side-arm was again isolated from the main reaction space, and then the reaction was commenced in the usual way. It should be noted that here again there is a volume correction necessary when comparing the pressure decrease under illumination and that produced by the absorbent.

Details of such a run, and also the determination of water vapour by absorption in P_2O_5 are given in figure 22.

FIG. 21

RATE CURVE, RUN 17.



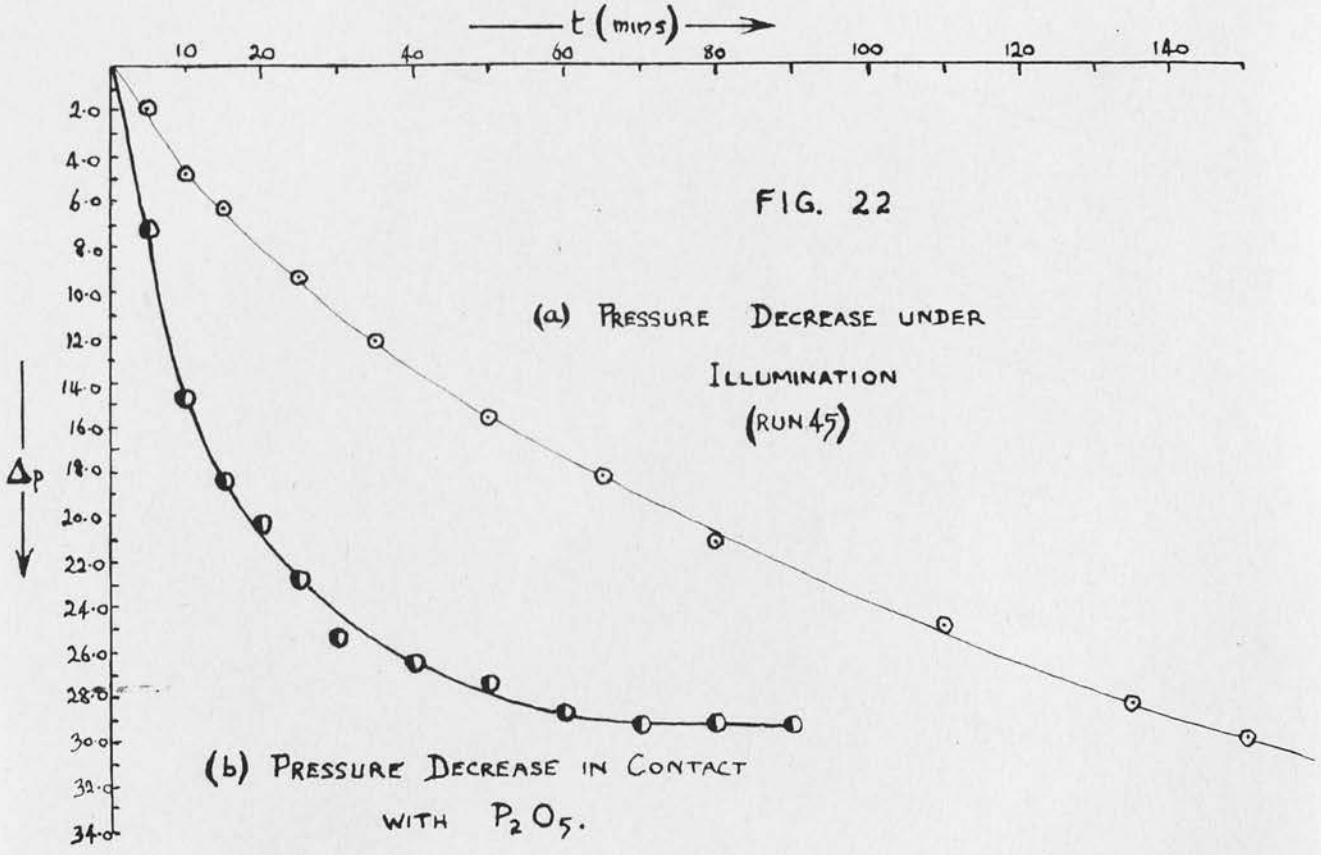
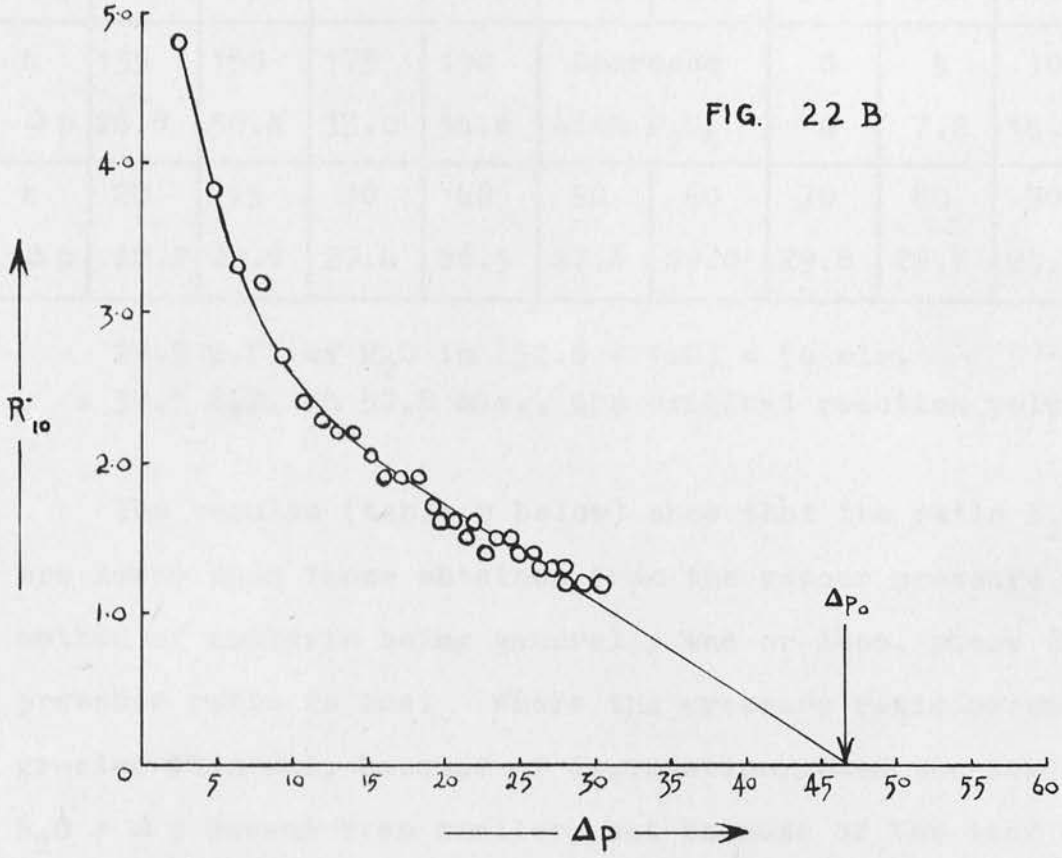


FIG. 22

(a) PRESSURE DECREASE UNDER ILLUMINATION (RUN 45)

(b) PRESSURE DECREASE IN CONTACT WITH P_2O_5.



RUN 45

RATE CURVE.

TABLE 8: Oxidation of spinach chlorophyll on TlBr, Run 45.

t	0	5	10	15	25	35	50	65	80	110
Δ p	0	2.2	4.8	6.4	9.4	12.2	15.6	18.2	21.2	25.0
t	135	150	175	190	Decrease		0	5	10	15
Δ p	28.8	30.4	33.0	34.6	with P_2O_5		0	7.2	14.8	18.4
t	20	25	30	40	50	60	70	80	90	
Δ p	20.2	22.8	27.4	26.5	27.8	29.0	29.8	29.8	29.9	

29.9 S.D. of H_2O in (52.8 + 1.2) = 54 mls.
= 30.5 S.D. in 52.8 mls., the original reaction volume.

The results (table 9 below) show that the ratio H_2O : Δp are lower than those obtained from the vapour pressure curve method of analysis being generally one or less, where the pressure ratio is one. Where the pressure ratio became greater than one, because of degradation, then the ratio H_2O : Δp became even smaller, but because of the lack of reproducibility in the results, it was impossible to derive any relationship between the increase in the pressure ratio and the decrease in the water ratio.

If the H_2O : Δp ratios greater than one obtained in the vapour pressure measurements are due to the removal of adsorbed water on evacuation, then it would not be entirely unexpected that the values as determined by means of P_2O_5 might be low. Water free in the gas phase will be removed

by the P_2O_5 and the resultant pressure decrease will be recorded on the gauge. Water that is reversibly adsorbed on the film and on the walls of the vessel, however, will distil to the P_2O_5 but will not show as a pressure decrease; the result by this method will therefore be low. It is also possible to show (as in runs 52 and 55) that after two or three hours contact with P_2O_5, the usual procedure for vapour pressure determination of the products will reveal water still present in the system. This is undoubtedly water which is adsorbed on the surface of the vessel or on the film or trapped or occluded by the film, and which will not be revealed by the P_2O_5. This water may be present either before the reaction or be produced by it; as the pressure ratio is constant and reproducible it would appear that it is more likely to be there initially, possibly as water of hydration, than that it is formed during the reaction; although some of that formed during the reaction must be adsorbed to give low P_2O_5 results. It has been suggested that solvent released from the film may compensate for this loss by adsorption to keep the pressure ratio constant.

As the results are never much greater than one, the middle fraction of the vapour pressure curves cannot be acetone, which would be taken up by the P_2O_5, also in those runs evacuated after contact with P_2O_5 the middle fraction is found to be still present.

TABLE 9: determination of water by means of P_2O_5 :-

Run	Pressure decrease	Pressure ratio	%age. reaction	H_2O by P_2O_5	H_2O by V.P.	$\frac{H_2O}{\Delta p}$ by P_2O_5	$\frac{H_2O}{\Delta p}$ total
40.	11.2	1.05	25	11.8	-	1.05	1.05
41.	19.2	0.92	42	21.2	-	1.10	1.10
42.	21.0	0.92	27	14.2	-	0.68	0.68
43.	20.0	0.97	44	25.0	-	1.25	1.25
45.	34.6	0.98	76	30.5	-	0.88	0.88
48.	32.6	1.72	50	4.4	-	0.12	0.12
50.	14.8	1.30	53	7.0	-	0.47	0.47
51.	15.4	1.56	51	6.2	-	0.40	0.40
52.	9.8	1.04	88	4.4	6.8	0.45	1.15
	The following results were obtained on nettle chlorophyll, but are of interest here:-						
54.	14.2	1.26	78	9.1	-	0.64	0.64
55.	18.6	1.28	84	8.2	4.5	0.44	0.69

The percentage reaction is calculated as -

$$\frac{\text{pressure decrease under illumination}}{\text{extrapolated pressure decrease}} \times 100.$$

The amount of water revealed by the vapour pressure measurement after standing over P_2O_5 will naturally depend on how long adsorbed water has had to distil undetected into the absorbent, and so it is not to be expected that the sum of the water revealed by the two methods should be equal to the initial pressure decrease under illumination.

Photo-oxidation of phaeophytin:

To investigate the variation in the amount of water revealed by the P_2O_5 with the increase in the extrapolated pressure ratios some pure phaeophytin was prepared by the method described on p.33 and its reaction with oxygen examined in this system in a manner identical to the investigation of chlorophyll. The method will therefore not be dealt with in any further detail.

It has been previously established that illumination of solid films of phaeophytin in the presence of oxygen gives a pressure ratio of three. By analogy to the chlorophyll reaction, reaction in the presence of absorbents will show whether this is a true oxygen ratio, or is the resultant of an uptake of n molecules of oxygen followed by the release of (n - 3) molecules of gases taken up by the absorbents. Runs 31 and 32, carried out in reaction vessel 10C in the presence of P_2O_5, show that the true uptake is four molecules of oxygen, with the release of one molecule of a gas absorbed by the P_2O_5; this gas had been identified in the usual way as water (run 7). Determination of the water produced at any stage in the reaction should therefore show the pressure decrease under illumination to be three times the amount of water present. This suggests that the reaction in chlorophyll is common to itself and phaeophytin, and the additional uptake

of two molecules of oxygen in phaeophytin may be related to the removal of the magnesium atom.

It was found that certain of these runs lead to pressure ratios greater than three; the excess appeared to increase with time. In the same runs the quantity of water revealed by the P_2O_5 was very much less than that expected, assuming one molecule of water released by one molecule of phaeophytin oxidised. If, however, it is possible to assume that the increase in the pressure ratio is due to the removal of water from the system by adsorption, as occurs in runs carried out in the presence of P_2O_5, then the increase in the pressure ratio is exactly that required by the low values in the water determination. The table of results (table 10) below shows in addition to the normal figures, a column giving the time required for the given pressure decrease to occur; runs taking less than an hour and a half show normal pressure ratios. In a later section on phytol (P. 76) it appears that a certain discrepancy in the results can be overcome by assuming a constant fraction of the water produced is adsorbed on various surfaces; in view of the small quantities involved in these reactions (10^{-5} -10^{-6} M.) it seems possible that adsorption will follow this course rather than that involving the increase in amount adsorbed with time, as postulated above.

In an attempt to overcome the difficulties of both the vapour pressure and the absorbent methods of analysis, the two methods were used together (runs 36 and 37), as follows:

Run 36: at three points in the reaction, the side-arm containing P_2O_5 was connected to the reaction vessel and oxygen and gaseous products were pumped out through a liquid oxygen trap in the usual way for a vapour pressure determination, until the pressure in the reaction vessel had been reduced by half. The reaction space and gauge jacket were then immediately isolated again, the whole procedure being completed as quickly as possible. While the dark pressure decrease due to the P_2O_5 was being recorded, the condensed products were distilled to a vessel for storage for their subsequent analysis by the vapour pressure curve method. After at least one hour, or longer if the uptake of water by the P_2O_5 were still continuing at an appreciable rate, the oxygen pressure in the gauge and jacket was brought back to its original value, the side-arm was shut off from the reaction vessel, and the run recommenced by raising the shutter to illuminate the film. Thus the products at each point at which the reaction was interrupted were determined by two methods, each in itself subject to an experimental error of opposite kind, and therefore the true result should lie between the two.

The method for run 37 was slightly different in that

only half the total oxygen pressure was admitted to the side-arm before commencing the run, and therefore the first half of oxygen and products were pumped out for storage at each interruption for analysis before opening the side-arm tap. In addition the reaction vessel was allowed to stand for twenty minutes in the dark before sampling the products, to allow the gas phase to come to equilibrium.

Finally, the acceleration found after removal of the products either by evacuation or by absorption is always present, but is never as great as in the chlorophyll reaction; this may be explained by assuming that it is the same stage of the reaction which is reversing, and the effect is merely masked in phaeophytin by the greater uptakes.

TABLE 10: Oxidation of phaeophytin -

Run	Pressure decrease	%age reaction	Time of reaction	Pressure ratio	Water	Method	Water required
7	71.2	90	370 mins.	3.22	30	V.P.	23
28	169	75	320	2.98			
29	160	73	280	3.04			
30	169	75	300	3.17			
31	116	85	275	4.02	-	-	-
32	116	86	300	3.94	-	-	-
34	98	68	125	3.40	39	V.P.	33
35	133	82	245	3.66	14	P_2O_5	13
33	139	85	280	3.70	16	P_2O_5	13
36	(a) 32	24	30	3.04	2.0	V.P.	5.3
					3.2	P_2O_5	5.3
	(b) 45	-	60	-	3.4	V.P.	7.5
					5.4	P_2O_5	7.5
	(c) 30	-	60	-	-	-	-
					6.0	P_2O_5	5.0
37	(a) 33.4	28	30	2.96	6.8	P_2O_5	5.5
					-		-
	(b) 32.2	-	35	-	17.0	V.P.	5.5
					8.2	P_2O_5	5.5
	(c) 24.0	-	40	-	7.0	V.P	4.5
					3.0	P_2O_5	4.5

Runs 28, 29 and 30 were carried out in a special reaction vessel (Fig. 23) in which the film was supported on a sintered glass disc. By carefully depositing the pigment in the sinter, it was hoped that a reproducible film would be formed and give reproducible rate curves. The three curves are superimposed in Figure 24, and it can be seen that though the rates are of the same order, they are by no means identical. Because of the difficulty of depositing the film, (a stream of air was drawn through the vessel to dry the solution) and because no great improvement in reproducibility was achieved, this attempt was abandonned.

All quantities are expressed in scale divisions, and the figures under "water required" are calculated assuming the pressure decrease under illumination to be three times the water formed.

Run 33 in the above table is illustrated in Figure 25.

TABLE 11: Experimental data - Run 33.

t	0	2	5	10	15	20	30	35	40
Δp	0	3.0	7.8	15.0	22.2	28.8	39.2	44.4	48.8
t	45	50	55	60	65	70	80	90	100
Δp	53.2	56.0	59.8	63.4	66.6	69.6	75.8	81.2	86.4
t	110	120	130	140	150	160	170	180	190
Δp	91.0	95.6	99.4	103.2	107.0	109.8	113.6	117.0	119.8
t	200	210	220	230	240	250	260	275	280
Δp	121.4	124.0	126.2	128.4	130.6	132.6	135.0	137.8	138.8

Uptake of water by P_2O_5 :-

t	0	5	10	15	20	30	40	50
Δp	0	1.8	3.6	4.6	5.6	7.2	8.6	10.0
t	70	80	90	100	110	120	140	160
Δp	11.6	12.2	13.0	13.8	14.9	15.6	16.0	16.1

FIG. 23

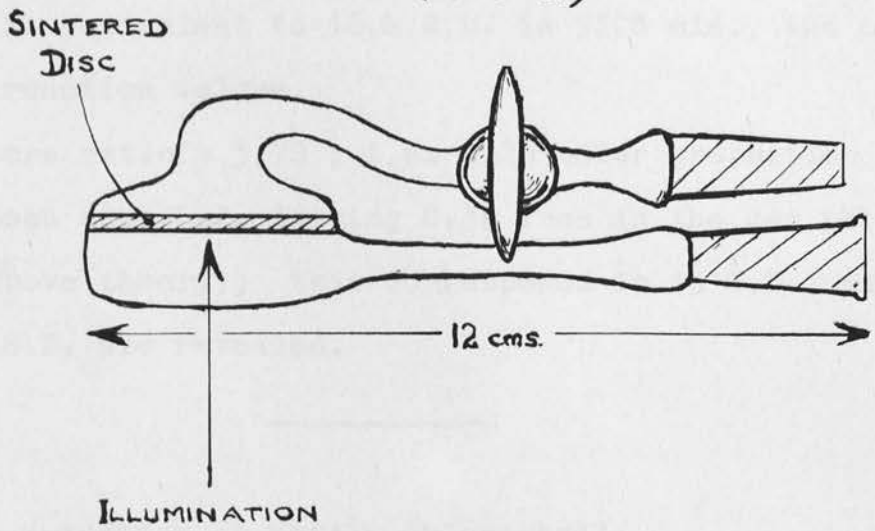
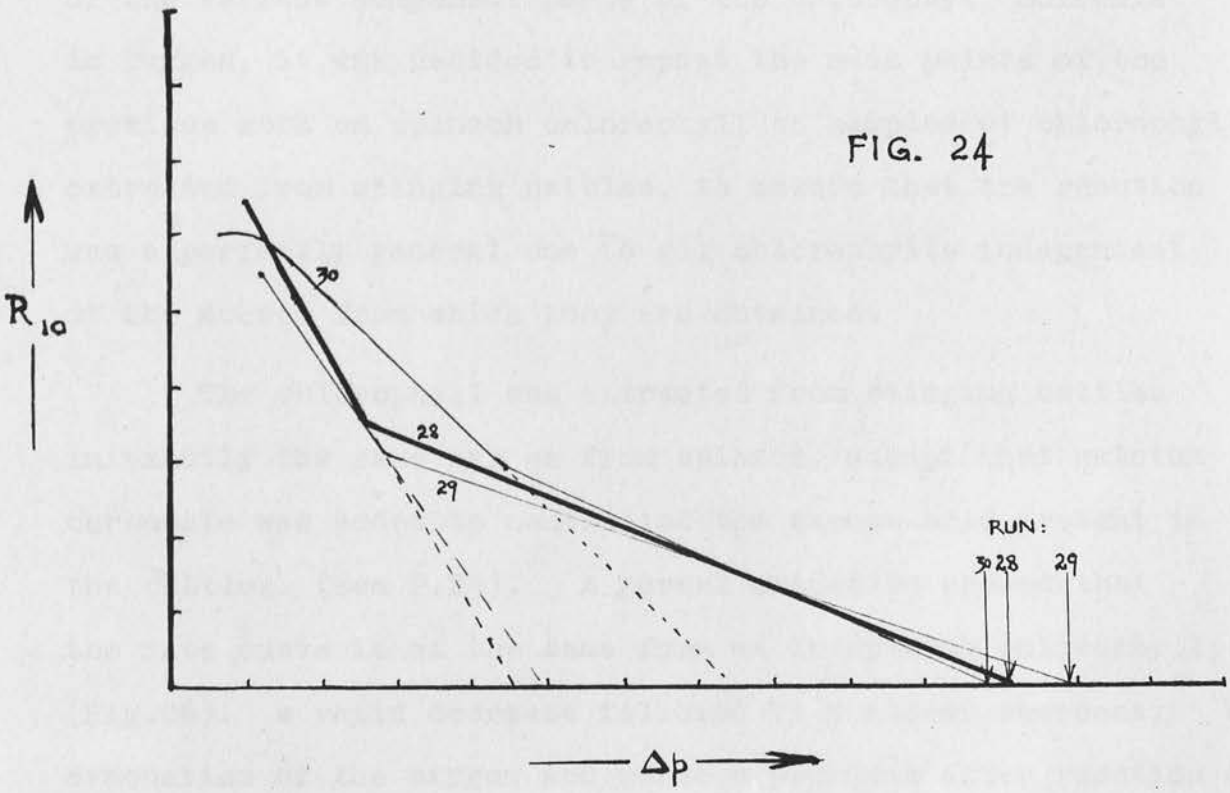

SINTERED
DISC
12 cms.
ILLUMINATION

FIG. 24


R_{10}
30
28
29
RUN:
30 28 29
Δp

Now 16.0 S.D. in (52.8 + 1.2) = 54.0 mls.
is equivalent to 16.4 S.D. in 52.8 mls., the original reaction volume.

Pressure ratio = 3.70 ; i.e. 0.70 molar proportion of water has been adsorbed, leaving 0.30 free in the gas phase (on the above theory); this corresponds to 13 S.D., experimentally 16.4 S.D. are revealed.

Photo-oxidation of nettle chlorophyll:

Before proceeding to investigate the photo-reactions of the various component parts of the chlorophyll molecule in oxygen, it was decided to repeat the main points of the previous work on spinach chlorophyll on samples of chlorophyll extracted from stinging nettles, to ensure that the reaction was a perfectly general one to all chlorophylls independent of the source from which they are obtained.

The chlorophyll was extracted from stinging nettles in exactly the same way as from spinach, except that calcium carbonate was added to neutralise the excess acid present in the nettles. (see P.29). A normal oxidation showed that the rate curve is of the same form as in spinach chlorophyll; (Fig.26). a rapid decrease followed by a slower decrease; by evacuation of the oxygen and gaseous products after reaction or by removal of water by P_2O_5 an accelerated rate was obtained on

re-illuminating in oxygen. The pressure ratios, however, were not unity, but 1.10 - 1.30, indicating that a certain amount of degradation had taken place, in spite of the added calcium carbonate.

The quantity of water revealed in the usual experiments is shown below; the values are much the same as those obtained in spinach chlorophyll experiments, and are in approximate unit ratio to the pressure decrease under illumination. Thus the essential features of the reaction appear to be general to the chlorophyll molecule, independent of the source from which it is obtained.

A rate curve for a run on nettle chlorophyll is given in Figure 26, the experimental data are in table 12, and a general summary of the results is in table 13.

TABLE 12: (run 54 - experimental data).

t	0	5	15	20	25	30	42	50	62
Δp	0	1.6	3.1	3.8	4.4	5.0	6.6	7.1	8.0
t	70	85	115	130	145	160	175	190	
Δp	8.9	9.9	11.6	12.2	13.0	13.9	14.3	15.0	

TABLE 13

Run	Pressure ratio	Pressure decrease	Water	Method	$\frac{H_2O}{\Delta p}$	$\frac{H_2O}{\Delta p}$ *
53	1.12	11.2	9.2	V.P.	0.82	0.89
54	1.28	15.0	8.5	P_2O_5	0.57	0.78
55	1.28	18.6	8.2	P_2O_5	0.44	0.78
			5.9	V.P.	0.32	
56	1.10	8.2	6.4	P_2O_5	0.78	0.91

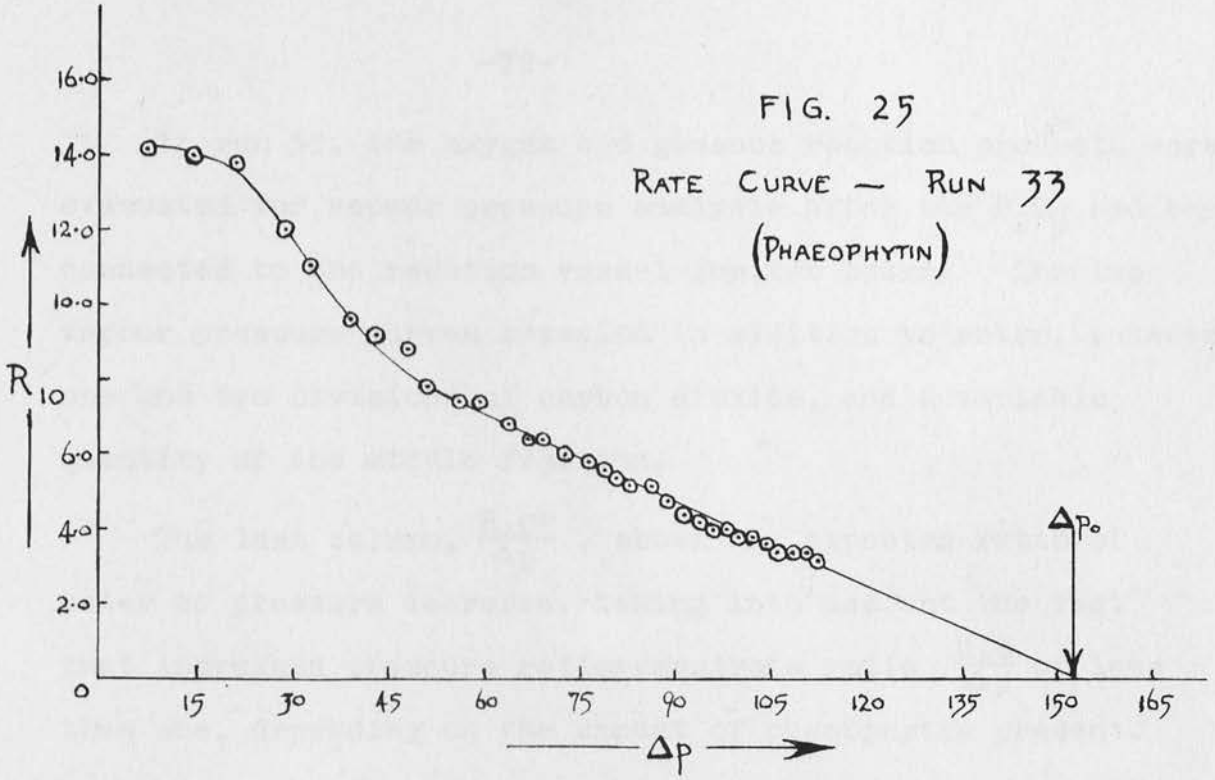


FIG. 25

RATE CURVE — RUN 33

(PHAEOPHYTIN)

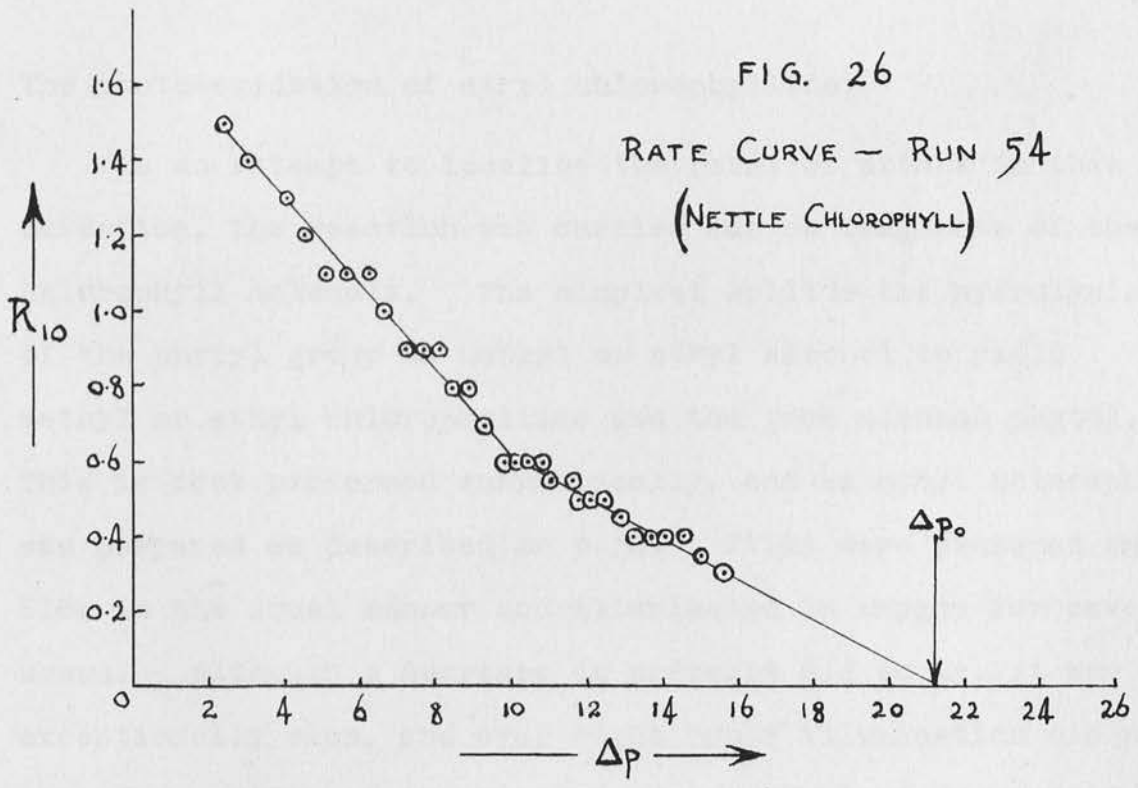


FIG. 26

RATE CURVE — RUN 54

(NETTLE CHLOROPHYLL)

In run 55, the oxygen and gaseous reaction products were evacuated for vapour pressure analysis after the P_2O_5 had been connected to the reaction vessel for two hours. The two vapour pressure curves revealed in addition to water, between one and two divisions of carbon dioxide, and a variable quantity of the middle fraction.

The last column, $\frac{H_2O^*}{\Delta p}$, shows the expected ratio of water to pressure decrease, taking into account the fact that increased pressure ratios require a ratio $\frac{H_2O}{\Delta p}$ of less than one, depending on the amount of phaeophytin present.

The photo-oxidation of ethyl chlorophyllide:

In an attempt to localise the point of attack in this oxidation, the reaction was carried out on fragments of the chlorophyll molecule. The simplest split is the hydrolysis of the phytyl group in methyl or ethyl alcohol to yield methyl or ethyl chlorophyllide and the free alcohol phytol. This is best performed enzymatically, and so ethyl chlorophyllide was prepared as described on p.34. Films were prepared on TlBr in the usual manner and illuminated in oxygen for several hours. Although a decrease in pressure did occur, it was exceptionally slow, and over eight hours' illumination did not exceed one fifth of the total decrease required for a unit pressure ratio. It was not possible to construct rate

curves and extrapolate a total pressure decrease at zero rate because of the slowness of the reaction. Figure 27 shows the form of the pressure decrease curves: after an initial appreciable decrease, the curve becomes very much flatter and shows a more or less continuous uptake at a very slow rate. As thallous bromide itself is known to take up oxygen under illumination (57), two blank runs were carried out on this substrate in 100 mm. of oxygen and the decrease compared with that found on ethyl chlorophyllide. Although it has previously been decided that no correction for this uptake by the thallous bromide was necessary in chlorophyll runs, it is seen that the two effects here are by no means incomparable, and that in fact an appreciable proportion of the decrease found in ethyl chlorophyllide films may be attributed to the uptake of oxygen by the substrate. Because of the slowness of runs carried out on glass substrates, all ethyl chlorophyllide runs were on TlBr.

If the observed pressure decrease is due to reaction of the ethyl chlorophyllide, then it appears to be very incomplete and proceeding at very slow speeds, and therefore very unlike the reaction of the parent molecule, phytyl chlorophyllide.

It is more likely that it is in fact a reaction of an

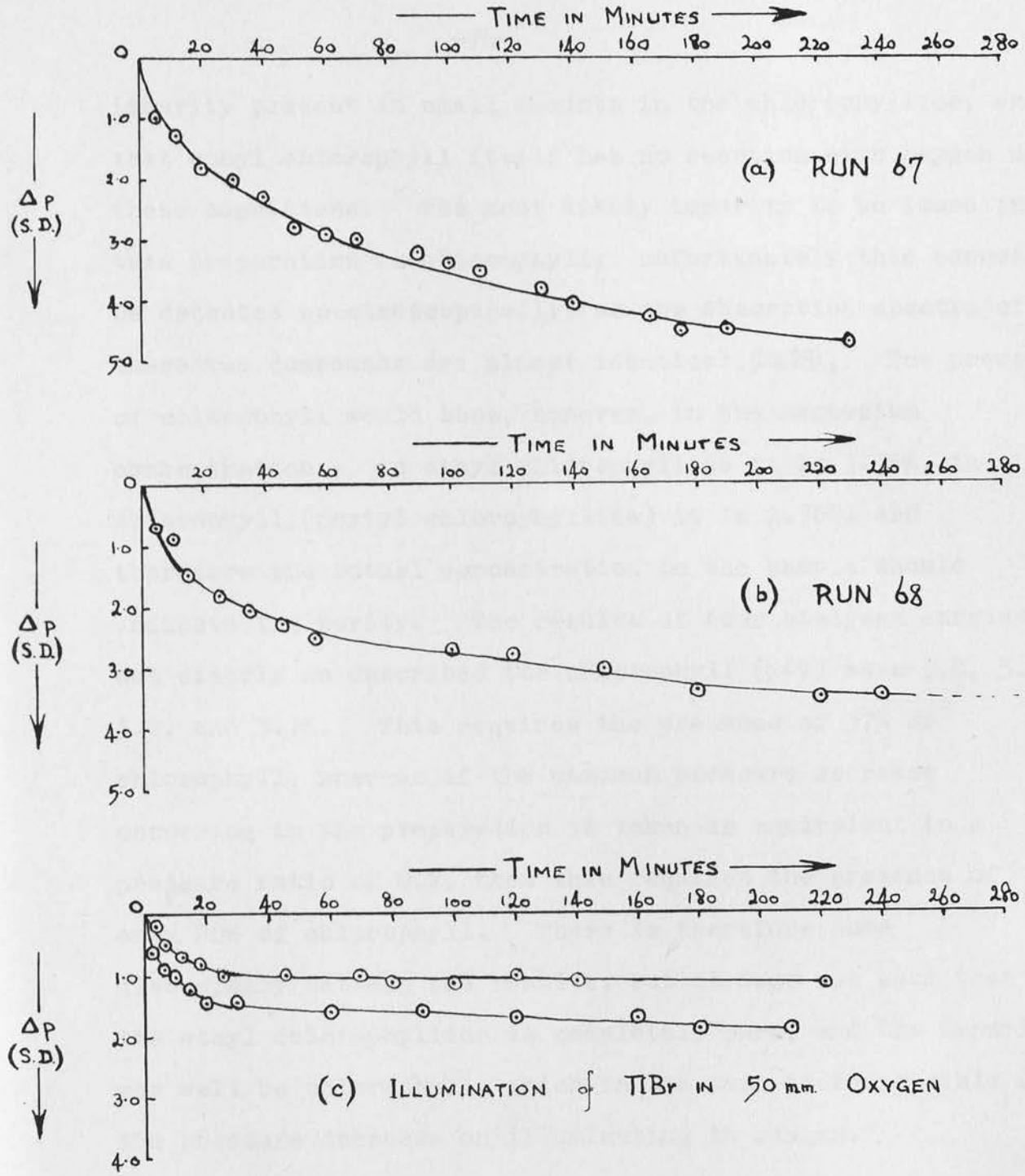


FIG. 27

PHOTO-OXIDATION of ETHYL CHLOROPHYLLIDE

impurity present in small amounts in the chlorophyllide, and that ethyl chlorophyll itself has no reaction with oxygen under these conditions. The most likely impurity to be found in this preparation is chlorophyll; unfortunately this cannot be detected spectroscopically, as the absorption spectra of these two compounds are almost identical (fig. 29). The presence of chlorophyll would show, however, in the magnesium concentration:- in ethyl chlorophyllide it is 3.75%, in chlorophyll (phytyl chlorophyllide) it is 2.70%, and therefore the actual concentration in the sample should indicate the purity. The results of four analyses carried out exactly as described for chlorophyll (p.49) were 3.0, 3.3, 3.0, and 3.1%. This requires the presence of 37% of chlorophyll, whereas if the maximum pressure decrease occurring in the preparation is taken as equivalent to a pressure ratio of 0.2, then this requires the presence of only 20% of chlorophyll. There is therefore some discrepancy between the results, but it does not seem that the ethyl chlorophyllide is completely pure, and the impurity may well be chlorophyll, which in its turn is responsible for the pressure decrease on illuminating in oxygen.

It may be concluded therefore that ethyl chlorophyllide has no reaction with oxygen comparable to that of phytyl chlorophyllide under the same conditions.

After each run, the films were extracted qualitatively

for their absorption spectra and also for examination by paper chromatography, as described on p.40

The chromatograms indicated a breakdown of the molecule, even though the conclusion reached above is that the pigment did not react. Blank runs, however, show that more than the number of starting spots can be obtained in the dark, and possibly also in vacuum. These results will be discussed in relation to the extracted films of chlorophyll in a later section. The results are tabulated below (table 15), a typical chromatogram is shown in figure 28, and the absorption spectra are shown in figure 29.(58)

TABLE 14: Experimental data, illumination of ethyl chlorophyllide in oxygen; also blank runs on TlBr.

Run 67 -

t	0	7	12	20	30	40	50	60	70	90
Δp	0	1.0	1.3	1.8	2.0	2.3	2.6	2.9	3.0	3.2
t	100	110	130	140	165	175	215	Run 68		0
Δp	3.3	3.5	3.8	3.9	4.3	4.5	4.7			0
t	5	10	15	25	35	45	55	100	120	150
Δp	0.7	0.8	1.5	1.8	2.1	2.3	2.5	2.7	2.8	3.0
t	180	220	230	280	300	Run 72			0	3
Δp	3.4	3.5	3.5	3.7	3.9	(Blank)			0	0.2
t	6	13	18	25	30	45	60	90	120	180
Δp	0.5	0.7	0.8	1.0	1.0	1.0	1.1	1.0	1.0	1.1
t	Run 66		0	2	7	10	15	20	25	30
Δp	(Blank)		0	0.6	0.9	1.0	1.2	1.4	1.4	1.4
t	60	70	90	105	150	195				
Δp	1.6	1.5	1.6	1.6	1.7	1.8				

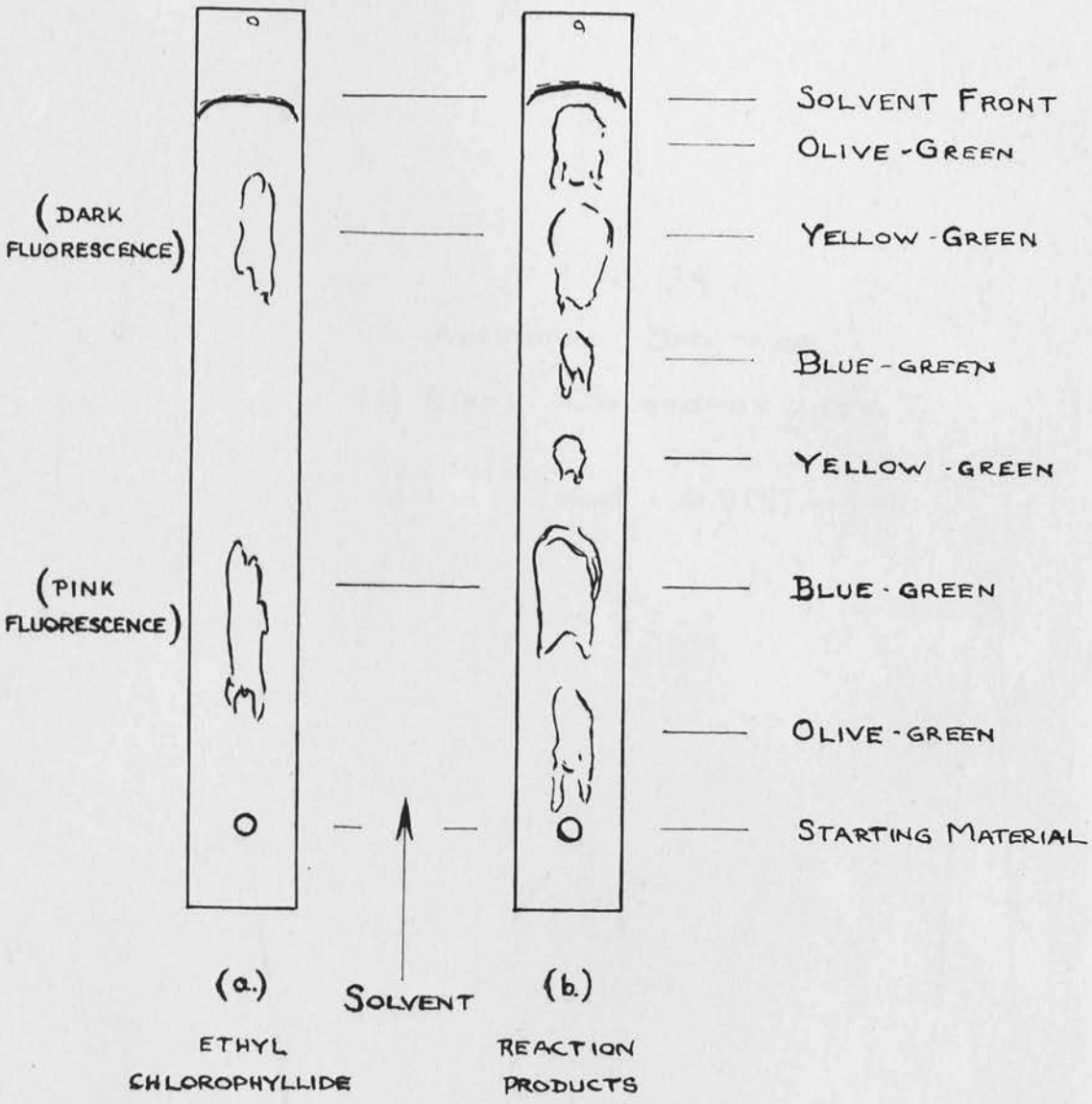


FIG. 28

PAPER CHROMATOGRAPHY OF

ETHYL CHLOROPHYLLIDE.

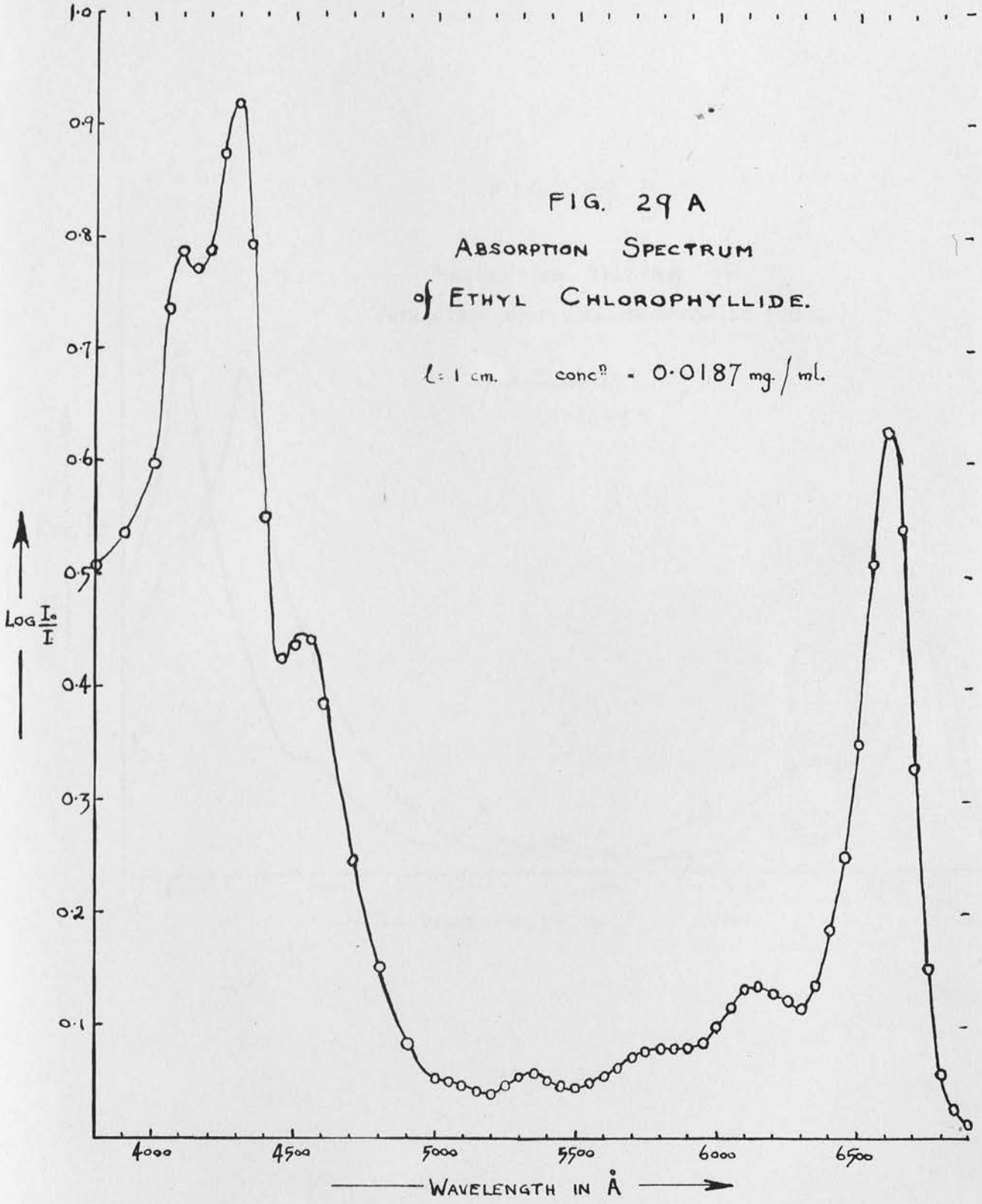


FIG. 29 A

ABSORPTION SPECTRUM

of ETHYL CHLOROPHYLLIDE.

l = 1 cm. conc^{n} = 0·0187 mg./ml.

FIG 29 B

ABSORPTION SPECTRA OF
EXTRACTED ETHYL CHLOROPHYLLIDE FILMS.

COMPARE CURVE 'A' WITH
FIG. 29 A

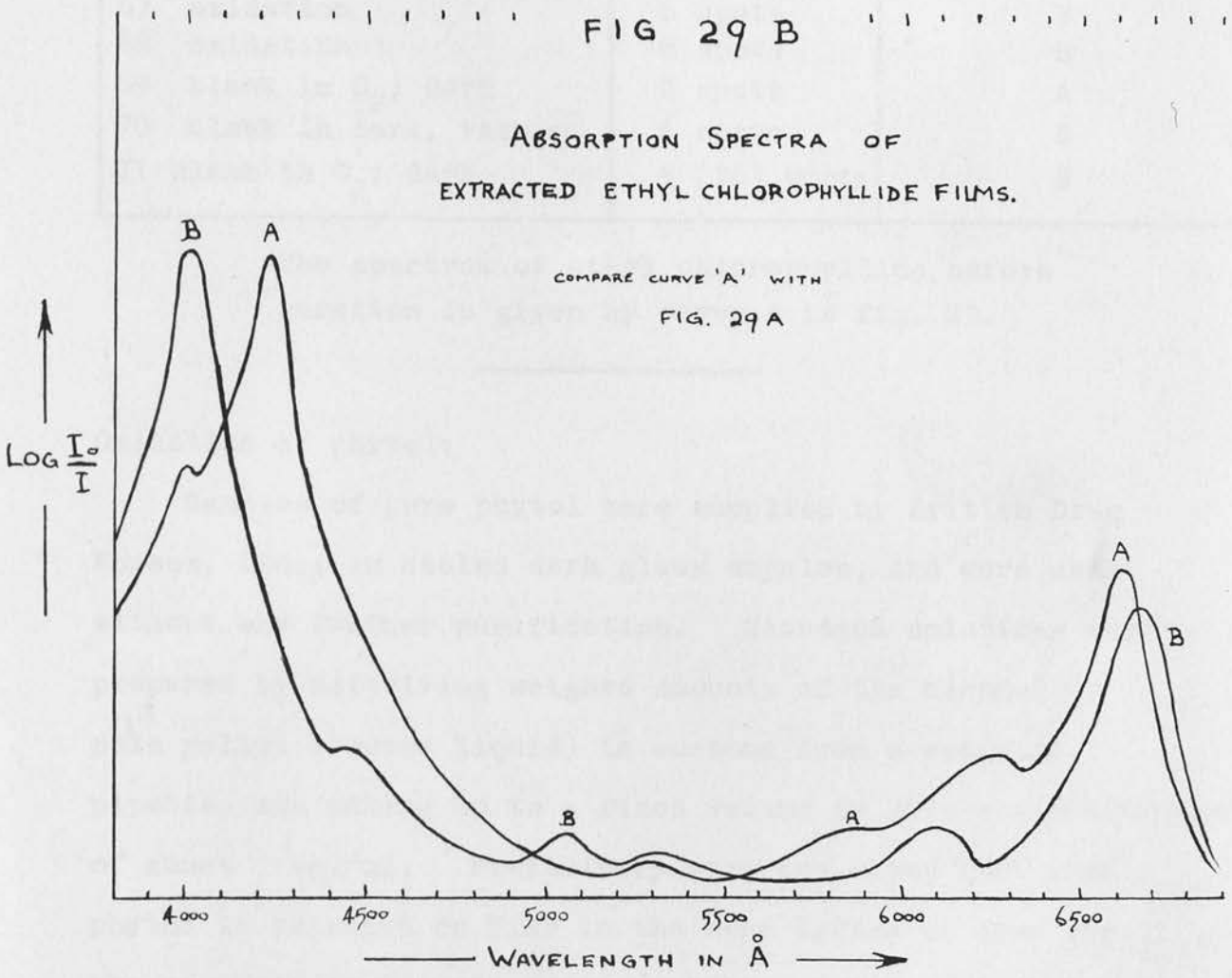


TABLE 15:

Run	Result of Chromatogram	Absorption spectrum (fig.29, A or B)
67 oxidation	6 spots	B
68 oxidation	6 spots	B
69 blank in O_2; dark	2 spots	A
70 blank in dark, vacuum	6 spots	B
71 blank in O_2; dark	5 (?6) spots	B

The spectrum of ethyl chlorophyllide before reaction is given by curve A in fig. 29.

Oxidation of phytol:

Samples of pure phytol were supplied by British Drug Houses, Ltd., in sealed dark glass ampules, and were used without any further purification. Standard solutions were prepared by dissolving weighed amounts of the alcohol (a pale yellow viscous liquid) in acetone from a weighing pipette, and making up to a fixed volume to give a concentration of about 1 mg./ml. Preliminary work has shown that when phytol is oxidised on TlBr in the same system as used for the previous work, unit pressure ratios can be obtained, but only with difficulty and not consistently. It was thought that the low values obtained were due to lack of contact between phytol and the substrate. If this is so, then it may be possible that by increasing the amount of substrate, the pressure ratio would increase. The results summarised

in table 16 below show that this is not so in practice, and that if anything, the reverse tends to happen. As the film is prepared in an identical way to the chlorophyll films, where the pigment can be seen and is, visually at least, all on the substrate, then it would not be expected that up to 50 or even 70% of the phytol would be out of contact with the substrate, as suggested by the pressure ratios. Lack of contact would not therefore seem to be the reason for the irreproducible values obtained.

A method was developed for the analysis of the small amounts of phytol in use, to attempt to explain these irreproducible ratios. It was based on the simple reaction of bromine with the double bond in phytol, as detailed on p.39. The results on extracted films show a rough correlation with the quantity of phytol which would be expected to remain on the film after illumination on the basis of the oxygen uptake curves. For example, in run 78, the extrapolated pressure decrease was 20.2 S.D., (corresponding to a pressure ratio of 0.23) the pressure decrease under illumination was 9.4 S.D.; the percentage reaction on this basis is therefore -

$\frac{9.4}{20.2} \times 100 = 47\%$; analysis showed 0.98 mg. of phytol remaining out of the original 1.72 mg.; this represents $\frac{1.72 - 0.98}{1.72} \times 100 = 43\%$ reaction. Other results are given in table 16.

This correlation suggests that, at least from 40 to 80% reaction, some effect, constant during each run, is upsetting the pressure measurements, so that there is consistent error occurring in the rate curves.

The commonest sources of error found in this system have been those due to the sorption of water vapour, and vapour pressure curves on the products of this reaction have shown that water is produced (run 58). These curves also show that no carbon dioxide is produced and only a little of the middle fraction, (one or two divisions) and which in this case can probably be attributed to the acetone-deposited films. It is possible therefore that here too the irreproducibility is due to the sorption of varying amounts of water; so that as the conditions change from film to film, the resultant rate of apparent pressure decrease will also change. If the fraction of water adsorbed is constant, however, the rate curve will increase by a constant fraction, so that although the absolute values of the pressure ratios will be incorrect, the qualitative form of the curve will still be valid. If this is the case, then the only reliable data will be furnished by runs performed in the presence of P_2O_5, as here the variable, the extent of removal of water, should be constant at 100%. The first run carried out in this manner (run 61) gave a pressure ratio of 0.71, approximately twice the "normal" ratio. Two other runs

(81 and 82), gave pressure ratios of 0.95 and 0.98; these are (experimentally) good unit ratios, and show that there is one molecule of oxygen taken up in the oxidation of one molecule of phytol. In the absence of P_2O_5, however, the pressure ratio is not so great, indicating that some gas (water) is given off in variable amounts. It would be more reasonable to assume that 'n' molecules of water are constantly given off, where n is some integer, but owing to considerable and variable extents of adsorption, the net pressure decrease is not (1 - n), but (1 - n + x), where x is the fraction of the released water removed from the gas phase by adsorption. Since a pressure increase has never been observed, (n - x) must always be less than one; this means that when n is greater than one, then 'x' the fraction of water removed by adsorption, must not be less than (n - 1)/n. Instead of imposing this arbitrary condition on the system, it is simpler to consider the case where n = 1, and compare the variation in the pressure ratio and in the ratio $\frac{H_2O}{\Delta p}$ with the possible variation in the values of 'x'. This is suggested by the fact that in this case the ratio $H_2O / \Delta p$ is not infrequently greater than one, instead of the more usual values of less than one found in chlorophyll.

The graph of X the pressure ratio, against $y = H_2O/\Delta p$: X for values of the water adsorbed between 0.1 and 0.9 shows

that although none of the experimentally derived points lies on the curve, they do follow the general trend. The three points lying at the highest pressure ratios, 0.71, 0.94 and 0.98, from runs carried out in the presence of P_2O_5, are assumed to have the ratio $H_2O : \Delta p = 0$ as no free water should remain in the reaction space after contact with P_2O_5 for a suitable length of time (Figure 30).

Thus the most likely course for the reaction is the uptake of one molecule of oxygen followed by the release of one molecule of water, the experimentally observed pressure decrease being due solely to the adsorption of water. There is no obvious reason why the adsorption should occur to such a great extent in this case and not in the case of chlorophyll.

The oxidation of phytol supported on a glass substrate does not conform to these ideas; for the first time there appears to be a fundamental difference in the reactions on the two substrates. Runs 63 and 75 on glass show that no water whatever is revealed by P_2O_5, and the water revealed by the vapour pressure curve method is no greater than the amount by which the usual unit ratio ($H_2O : \Delta p$) is usually exceeded. After each period of illumination in run 63, the P_2O_5 side-arm was connected to the reaction space in the usual way for one or two hours, but as no decrease was recorded,

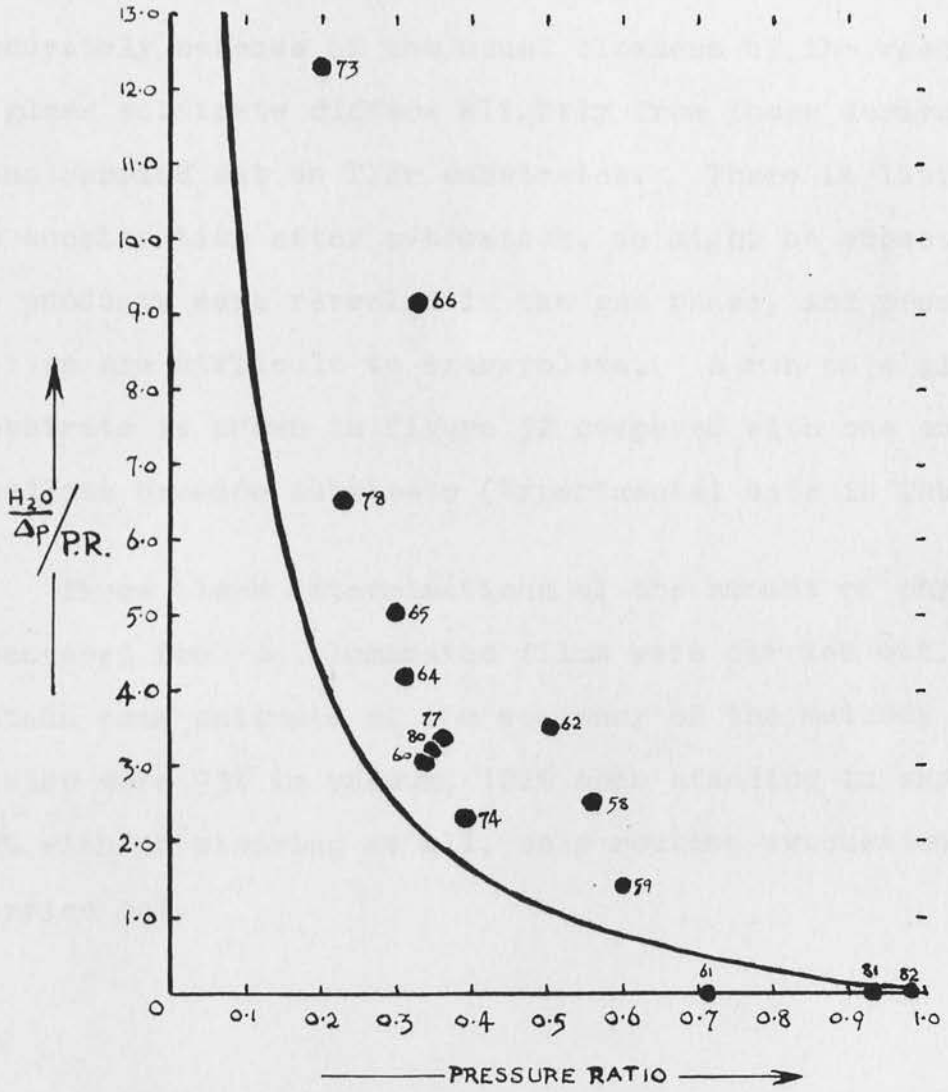

13·0
12·0
11·0
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
H2O/ΔP P.R.
PRESSURE RATIO
73
66
78
65
64
77
80
62
62
58
74
59
61
81
82

FIG. 30

the gases in the reaction space were evacuated in the usual way for the vapour pressure determination of any condensables. The form of the rate curve, although difficult to determine accurately because of the usual slowness of the reaction on a glass substrate differs slightly from those derived from runs carried out on TlBr substrates. There is little or no acceleration after evacuation, as might be expected when no products were revealed in the gas phase, and pressure ratios are difficult to extrapolate. A run on a glass substrate is shown in figure 32 compared with one on a thallous bromide substrate (Experimental data in Table 17).

Three blank determinations of the amount of phytol recovered from unilluminated films were carried out, to obtain some estimate of the accuracy of the method; the yields were 93% in vacuum, 102% when standing in oxygen, and 98% with no standing at all, only routine evacuations being carried out.

TABLE 16:

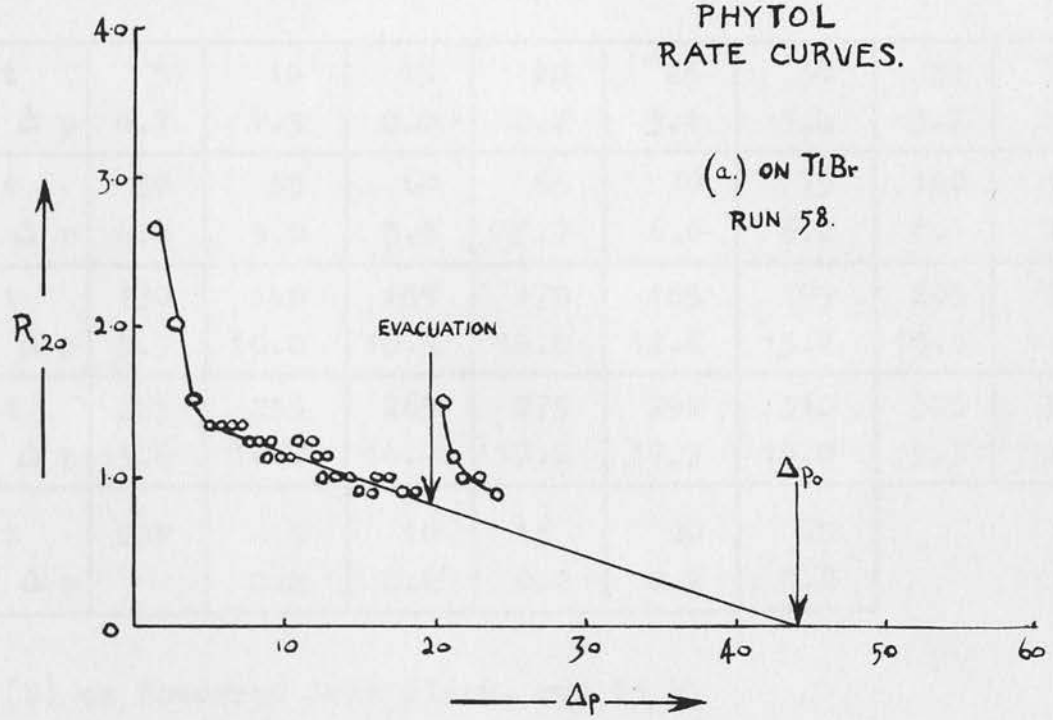
Run	$\frac{\text{Phytol}}{\text{TlBr}} \times 10^3$	Pressure ratio	$\frac{H_2O}{\Delta p}$	Method	$\frac{\Delta p}{\Delta p_o} \times 100$ %	Percentage phytol oxidised (analysis)
58.	9.7	0.56	1.43	V.P.	45	-
59.	8.2	0.60	0.82	P_2O_5	62	-
60.	0.4	0.34	1.01	P_2O_5	86	-
61.	0.4	0.71	-	-	- ...	carried out in the presence of P_2O_5.
62.	8.2	0.50	1.75	P_2O_5	25	-
64.	16.4	0.31	1.30	P_2O_5	68	-
65.	9.7	0.40	2.13	P_2O_5	100 ...	(extrapolated decrease equalled actual decrease.)
66.	9.7	0.33	3.05	P_2O_5	>100 ...	(Δp = 21.3 S.D. Δp_o = 20.0 S.D.)
73.	9.7	0.21	2.61	P_2O_5	67	-
74.	9.7	0.38	0.85	P_2O_5	51	-
77.	10.1	0.36	1.20	P_2O_5	55	84
78.	10.1	0.23	1.44	P_2O_5	47	43
79.	15.2	----	-	-	53	47
80.	5.0	0.35	1.10	P_2O_5	97	61
81.	5.0	0.93	-	-	96	81.
82.	5.0	0.98	-	-	66	-

and two runs carried out on a glass substrate:-

63.	-	0.3	(a) 0.04	P_2O_5	68	-
			0.46	V.P.		
			(b) 0.11	P_2O_5		
			0.17	V.P.		
			(c) 0.13	P_2O_5		
			0.00	V.P.		
75.	-	0.28	(a) 0.00	P_2O_5	43	-
			(b) 0.10	P_2O_5		

FIG. 31

PHYTOL
RATE CURVES.



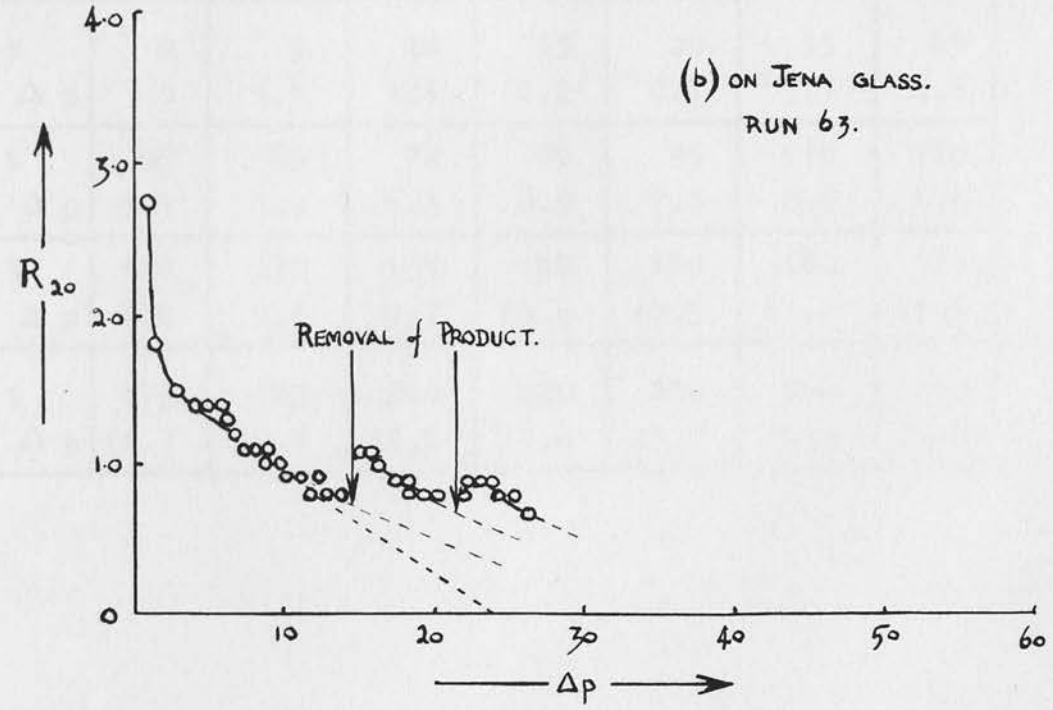


TABLE 17: Experimental data for phytol runs:-

(a) on TlBr, run 58 -

t	5	10	15	20	25	30	35	40	45
Δ p	0.7	1.3	2.0	2.7	3.1	3.4	3.7	4.0	4.3
t	50	55	60	65	70	75	100	110	120
Δ p	4.8	5.0	5.3	5.7	6.0	6.2	8.1	8.6	9.2
t	130	140	155	170	185	195	205	225	235
Δ p	9.5	10.0	10.9	12.0	12.8	13.2	13.8	14.8	15.2
t	245	255	265	275	290	310	325	335	
Δ p	15.6	16.2	16.7	17.2	17,9	19.0	19.3	19.7	
t	OFF	5	10	15	20	25			
Δ p		0.2	0.2	0.2	0.2	0.2			

(b) on Powdered Jena Glass, run 63 -

t	0	5	10	15	20	35	45
Δ p	0,	1.5	1.8	2.2	2.7	3.7	4.6
t	55	65	72	80	95	110	120
Δ p	5.1	5.9	6.3	6.9	7.7	8.6	8.8
t	120	130	135	140	150	160	170
Δ p	8.8	9.6	9.7	10.0	10.5	11.0	11.6
t	175	185	200	220	230	240	250
Δ p	11.7	12.1	12.8	13.6	13.7	14.3	14.6

DISCUSSION.

Previous work on the photo-oxidation of solid films of chlorophyll has been carried out on samples of copper stabilised chlorophyll, in which magnesium is partly replaced by copper, (one atom in ten); copper being a better co-ordinating atom than magnesium, the effect of this substitution is to stabilise the preparation, the fluorescence is quenched, the phase test is negative, and solid and solutions become more resistant to bleaching under illumination.

Illumination of such samples in the presence of oxygen results in a pressure decrease occurring in unit ratio to the chlorophyll present; the gas phase products of the reaction were believed to be water, carbon dioxide, and acetone, with the water predominating, while the solid residue contained a peroxide group again in unit ratio to the oxidised chlorophyll.

The work described in this thesis has been concerned with the comparison of these results with similar studies on plant chlorophyll, freshly extracted from various sources.

The initial difficulty of the increasing and irreproducible ratios was shown to be due to the loss of the magnesium atom, with the ultimate formation of phaeophytin. This did not occur in copper stabilised samples, and was an indication of the difficulties to be expected in the use of plant chlorophyll. It was finally found necessary to

extract samples every eight to ten weeks, as after this the sample showed degradation, not only in the pressure ratio, but also in the absorption spectrum. The magnesium analyses showed that there was a direct correlation between the loss of magnesium and the increase in the uptake of oxygen.

With fresh chlorophyll a reproducible unit pressure ratio has been found on illumination in oxygen, as with copper chlorophyll. Determination of the quantity of water formed during the reaction has shown that it equals the initial pressure decrease under illumination, as far as can be judged considering the difficulties experienced with adsorption. The true oxygen uptake is therefore two molecules of ~~chlorophyll~~ oxygen, and the experimentally observed decrease is the resultant of this and the release of one molecule of water.

It has also been shown that ethyl chlorophyllide has no reaction comparable to that of the phytyl chlorophyllide under the same conditions, and that the slight pressure decrease obtained is probably due to the presence of chlorophyll as impurity. Phytol has a reaction closely allied to that of chlorophyll, but involving the uptake of only one molecule of oxygen and the release of one molecule of water; the additional molecule taken up in chlorophyll must therefore be affected by the phytol-chlorophyllide link.

These results have been obtained by pressure measurements on a system involving the uptake of oxygen and the release of water by films of pigment supported on thallous bromide or ground glass. Measurements in such a system must be complicated by adsorption of the vapour on the various surfaces; such adsorption has been demonstrated, and some anomalous results can be explained by making certain assumptions about the extent of this sorption. If the adsorption were constant, then the results obtained might be made accurate by application of some factor designed to compensate for the irregularities caused by the loss of water. The variation in the form of the rate curve, and the variation in pressure ratios found in certain cases, however, (notably phytol) show that not only is the adsorption taking place to different extents in different films, but that the rate of reaction itself varies from film to film.

The greatest errors occurring in this work probably arise not from irreproducible rates as such, but from this adsorption of water. In view of the small quantities involved, and because no exact correlation of the amount of water revealed by P_2O_5 (probably the most reliable method) with the extent of reaction was revealed, it would seem most likely that the amount adsorbed will be directly proportional to the amount produced, i.e. a constant fraction of the water released will be lost. In the experiments with phaeophytin,

it was postulated that the increase in the pressure ratio with time was due to the adsorption of water in increasing amounts. This may be due to the formation of multilayers in place of a monolayer on the phaeophytin film, or it may be due to the condensation in crevices in the film formed by the oxidation and not found in chlorophyll films. It has never been explained why the pressure ratio in runs on chlorophyll is normally very reproducible when the adsorption of water appears to vary from film to film. The fact that adsorption does take place in this system is seen from the results of the water determination by P_2O_5, which were always below the unit ratio required by the theory. Solvent is undoubtedly released by the film, (in one case, three scale divisions increase was recorded on standing a film in the dark evacuated overnight) but it is unreasonable to expect that exactly sufficient solvent would be released to compensate for the water lost by adsorption in every case, especially considering the variation in the rate curves, and therefore presumably in the structure of the film.

The variation in the rate curve extends from a straight line, through a straight line prefaced by an induction period, to a more complex curve composed of two straight line portions, this type occasionally with a slight induction period also;

this is shown in Figure 32, type (c) being the most common.

The following factors may affect the form of the rate curve:- (a) regularity of film; a uniform film will allow even intensity of illumination throughout, and all parts will therefore react at the same rate, whereas an irregular film will lead to shading of the back layers in the thicker parts, which will therefore react more slowly than the top layers; and (b) porosity of film - if the film is not porous to oxygen and the gaseous reaction products, then a rapid surface oxidation will take place followed by a slower internal oxidation governed by the rate of oxygen diffusion to the internal pores; outward diffusion of products may also compete with the oxygen for the space in the pores. If one stage of the reaction is reversible, as does seem likely, then any impedance or hindrance to the removal of product of the reversible stage may tend to affect the reaction. Reproducibility of the rate curve will involve both of these factors, and will also depend on a reproducible film area and depth.

A rate curve of the form shown in the figures (a) or (c) in which $\frac{d}{dt}.\Delta p$ decreases linearly with Δp, can be shown to be due to one or a succession of unimolecular reactions (59).

A feature of particular interest in the rate curve is

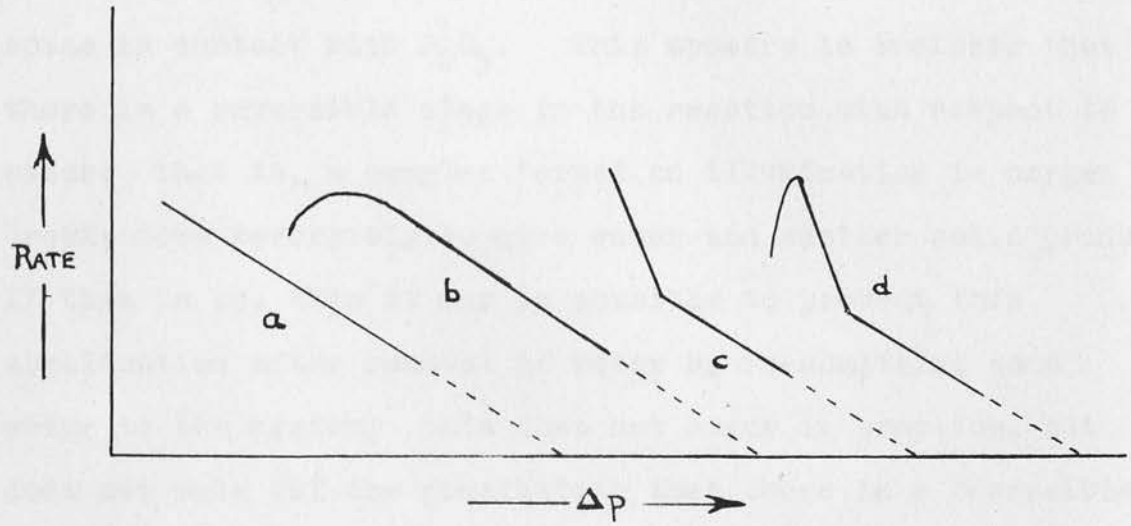

RATE
a
b
c
d
Δp

FIG. 32

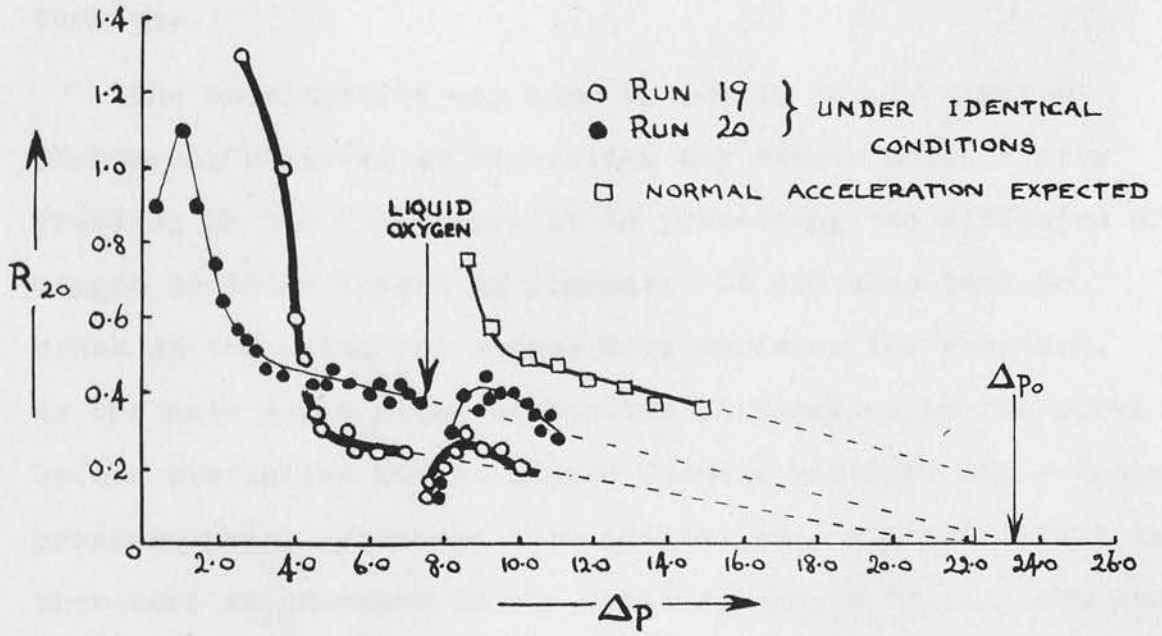

1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
R20
O RUN 19
● RUN 20
} UNDER IDENTICAL CONDITIONS
□ NORMAL ACCELERATION EXPECTED
LIQUID OXYGEN
Δp0
2·0
4·0
6·0
8·0
10·0
12·0
14·0
16·0
18·0
20·0
22·0
24·0
26·0
Δp

FIG. 33

the increase in the rate of reaction obtained on removing the gaseous products by evacuation, or by leaving the reaction space in contact with P_2O_5. This appears to indicate that there is a reversible stage in the reaction with respect to water; that is, a complex formed on illumination in oxygen breaks down reversibly to give water and another solid product. If this is so, then it may be possible to prevent this acceleration after removal of water by re-admitting some water to the system; this does not occur in practice, but does not rule out the possibility that there is a reversible stage in the reaction; unfavourable kinetics may prevent the back reaction - oxidised product + water ⟶ initial complex.

The acceleration may also be due to purely physical phenomena, however, as evacuation may remove product from crevices in the film where it is preventing the diffusion of oxygen to inner layers of pigment; it may also tend to break up the film, and expose more surfaces for reaction. As the rate curve after evacuation is parallel to the curve before evacuation but at higher values, however, the extrapolated pressure ratio increases with evacuation; the net effect is therefore an increase in the total uptake of oxygen, and not only in the rate of this uptake; this is more suggestive of a chemical than a physical phenomena, as the extrapolated

pressure ratio for a normal oxidation without evacuation is believed to represent 100% reaction of the chlorophyll present.

This acceleration has not been systematically examined in this work, but every feature previously reported has been confirmed. As the emphasis has been placed on pressure ratios, and therefore on accurate extrapolation, the oxidation was generally continued to at least 50% before removing products.

Although most of the experiments described have been carried out on a substrate of thallous bromide, almost every point can be reproduced in detail on inert supports such as powdered glass, so that the reaction is essentially one of the chlorophyll molecule, and the function of the thallous bromide is purely one of photosensitisor.

The initial rapid rate portion of the rate curve may be due in part to this photosensitisation. It is known that energy transfer between chlorophyll molecules is possible (60) and that energy transfer may also take place between triplets (61), thus initially the reacting molecules may not be those that are absorbing the light, or even those that are close enough to the photosensitisor to obtain energy from this source. Molecules therefore have two chances to react: by absorbing

light on their own or by having it transferred from one of the outside sources. As the length of the possible energy transfer path is not likely to be more than a few chlorophyll molecules long, however, the number of molecules able to react by having energy supplied from outside will drop very rapidly as the possible energy transfer paths are reduced by the ever increasing number of oxidised molecules, not able to transfer this energy. Thus the rate curve will show an initial rapid fall. The straight line rate curve is, on this basis, one from a film in which there is little transfer of energy, or one in which there is no value in the extent of oxidation at which the transfer drops abruptly, leaving only direct absorption. In the study of the photo-oxidation of rubrene in a similar system, Hochstrasser and Ritchie (39) found that there was a square relationship between the maximum rate expressed as Δp_{10} and the concentration of rubrene; this was taken to indicate the transfer of energy between two rubrene molecules. No such relationship has been found, however, in the present work. The acceleration period found occasionally is believed to be due to the reaction of chlorophyll molecules inside the film, possibly on the surface of the photosensitisor, with oxygen in the channels of the film; there will be a time lag due to diffusion of more oxygen into these channels before this reaction will be recorded as a pressure decrease; as all films are allowed to stand in

oxygen for 20 - 30 minutes before commencing the illumination, there will be initially an equilibrium pressure of oxygen in these channels.

The primary acts in the reaction are therefore the absorption of light and the subsequent electronic rearrangements taking place within the molecule, as described on page 12. -

(1) chlorophyll + $h\nu$ ⟶ chlorophyll *

(2) chlorophyll * ⟶ chlorophyll + $h\nu$

(3) chlorophyll * ⟶ chlorophyll ***

(4) chlorophyll *** ⟶ chlorophyll + $h\nu'$

Reaction one represents the absorption of a quantum of light by an individual chlorophyll, or possibly the absorption by transfer from the photosensitisor or from other excited chlorophyll molecules, in either case the result is the formation of a molecule excited to the first excited singlet level ('chlorophyll*'). This state may quickly revert to the ground state (reaction two) by the paths described in the introduction, or it may switch to the triplet level (reaction three), assuming this state to be present in chlorophyll. The present evidence is strongly in favour of this state, but it is not definite. The triplet molecule may also degenerate to the ground state (reaction four), but because of the longer life-time of this state, it may react with molecular oxygen, itself a triplet molecule in the ground state, and therefore

it reacts very readily with other triplets (reaction five below).

The exact mechanism of reaction five has not been elucidated but there are one or two possibilities: the excited pigment molecule may transfer an electron to an oxygen molecule adsorbed on the surface of the pigment to form the complex postulated by Weiss - $A^+O_2^-$ (23) where A represents the chlorophyll molecule; this will be facilitated by the fact that a triplet molecule, having two electrons with uncoupled spins, will tend to lose one electron easily; alternatively it may transfer the electron to the conduction band of the photosensitisor under suitable conditions, whence the adsorbed oxygen molecule may become O_2^- (62). Electrons may also be supplied by the photosensitisor, if they have been excited to the conduction band and a suitable acceptor molecule is present. It has been recently found in this department that the photoconductivity of thallous bromide is greater in vacuum than in the presence of some gases, notably oxygen and nitric oxide (63). This may be due to the transfer of current carriers from the conducting solid to adsorbed molecules of the gas. As negative oxygen ions form more easily than any positive oxygen ions, the transferred current carriers are most probably electrons.

Transfer of energy between two chlorophyll molecules has been postulated as being by some form of inductive

resonance, and not by the actual transfer of electrons, i.e. the electronically excited molecule may transfer the excess energy to a neighbouring molecule, possibly by an electro magnetic effect, to excite its neighbour to a higher electronic level.

Reaction 5 may be written as :-

(5) chlorophyll *** + $2O_2$ ⟶ A + H_2O

followed by:-

(6) A ⟶ B + H_2O

(7) B + O_2 $\xrightarrow{h\nu}$ final stable product, 'X'

Reaction five represents the formation of the initial oxygenated product; this in itself is probably a multistage reaction. This compound is then visualised as being in equilibrium with a second product 'B' and one molecule of water. This equilibrium is responsible for some of the features of the reaction not otherwise explained for example, the product of dissociation may be able to take up more oxygen on illumination and thus cause the increase in the pressure ratio after removal of water; as the two rates curves before and after removal are parallel, the quantum efficiencies of the two photo-reactions must be very similar. It may also explain the pressure below which no further decrease has been observed (37c); this will be the pressure of dissociation of

the compound A, and is about 0.05 mm. for spinach chlorophyll.

Thus in any normal reaction, i.e. one not interrupted by removal of product, reaction 5 is the sole cause of the pressure change, and explains why the large quantities of water required by the previous reaction scheme are not usually found, and also why there is no detectable variation in the amount of water revealed with the extent of reaction. If, however, the reaction is stopped at some point for the removal of water, then the equilibrium reaction 6 will come into operation; the formation of the second product B capable of taking up more oxygen on illumination will explain the increase in the extrapolated pressure decrease, and also possibly the larger quantities of water revealed by evacuation than by absorption in P_2O_5.

The scheme does not explain the presence of carbon dioxide and of the middle fraction in the gaseous products. The previous scheme visualised further reactions of A and B with the uptake of three and four molecules of oxygen and the release of water, carbon dioxide and acetone in the proportion required to maintain the unit ratio. It is thought, however, that this is unnecessarily complicated, and that as these large quantities of water have not been found, and that as the middle fraction is believed to be carbon tetrachloride, there is more likely to be a simpler explanation for the

presence of these compounds in the products. Carbon tetrachloride, for example, may come from the lubricating grease; taps were cleaned with CCl_4 before lubrication, and the reaction vessel ground glass joint was cleaned with this solvent before every run. As chlorophyll, and more particularly crystalline chlorophyllides, have been shown to possess zeolitic properties (64), with regard to water and carbon dioxide, it is not inconceivable that some CO_2 may be released from the film during reaction and thus appear as a product; it was usually found to be present in 0.05 to 0.10 molar proportions, slightly greater than the quantities previously reported from films of copper chlorophyll. Carbon dioxide was not found as a product from phytol, but did occur in one run on ethyl chlorophyllide. It may be also due to a partial breakdown of the molecule occurring as a side-reaction, as occurs when carotene breaks down slowly with the uptake of 12 moles of O_2, and the release of low molecular weight compounds including carbon dioxide (65). If carotene is oxidised in the same manner as chlorophyll has been in the present work, a pressure ratio of unity is obtained (69), unaffected by P_2O_5; and showing a similar reversibility. This is further evidence that the reaction is not concerned principally with the porphyrin nucleus.

The equilibrium reaction 6 may also be responsible for

the induction period found in some films, as the decomposition of A to the equilibrium value of the water vapour pressure will cause a pressure change opposed to the normal decrease. Attempts to reverse this reaction have failed. It had been reported by the earlier workers that if the products of illumination were frozen out in liquid oxygen for several hours, and then allowed to expand back into the reaction space, an accelerated rate was observed, similar to that found on removing the products by other ways; in the present work, however, two runs were interrupted by placing a refrigerant around a side-arm to the main reaction vessel; after several hours the refrigerant evaporated and the pressure in the system returned to within one telescope scale division of the original. Illumination then showed that the rate was not appreciably altered by this procedure (Fig. 33). It may be possible that in these two cases the conditions were such that reaction 6 was reversed. If this is so then it is further evidence that the acceleration is due to a real chemical effect and not to the physical conditions of the film.

Because of the hydration of chlorophyll (66), it may be difficult to distinguish in practice between the equilibrium of an oxygenated chlorophyll molecule (A) with a further product (B) and water, and the corresponding equilibrium between the one product A and two possible states of hydration; removal

of some of the water from the first hydrate may allow the molecule to take up more oxygen, thus increasing the pressure ratio after evacuation.

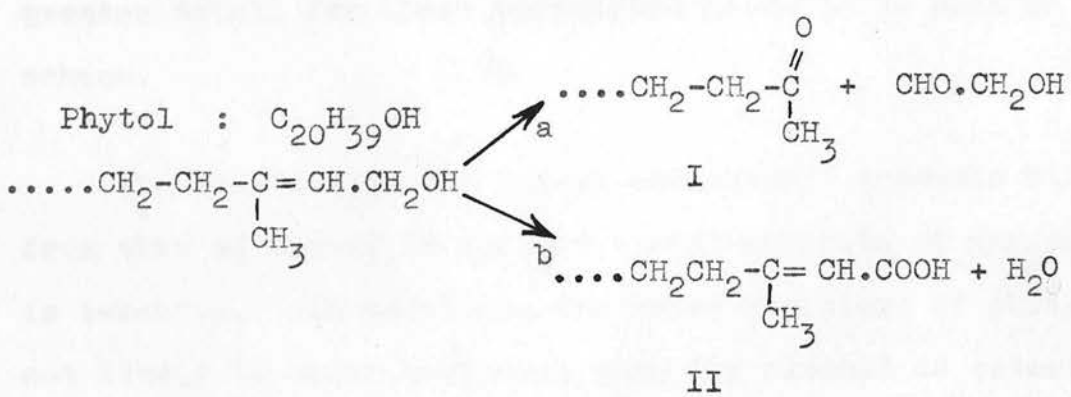
The point of attack of this reaction has not previously been investigated systematically. It has been known, however, that no large scale breakdown of the porphyrin ring is involved, as after 100% reaction the phase test is still positive. Allomerised chlorophyll has also been shown to react with oxygen under the same conditions in exactly the same manner; this rules out the possibility that the cyclopentanone ring is concerned in the reaction, in spite of the reactivity of the hydrogen atom on C_{10}. Hydrolysis of the molecule to split off the phytol group has given a valuable indication of the most likely point of attack - the residue of ethyl chlorophyllide does not have a reaction comparable to that of intact chlorophyll, whereas the phytol group does; this leads to the result that although the illumination is undoubtedly absorbed by the aromatic nucleus, the reaction takes place in the largest side chain. The mechanism of the energy transfer to this side chain is unknown, but is not likely to be transferred along the two $-CH_2-$ groups connecting the two parts of the molecule; as the side-chain is completely flexible, it may lie alongside the aromatic ring and thus receive its energy by some form of inductive resonance as postulated for the transfer of energy between two chlorophyll molecules. This would probably require

a frequency of vibration in phytol similar to one in chlorophyll if the transfer is to be at all efficient. The reaction of chlorophyll is not however identical to that of phytol. The former involves the uptake of two molecules of oxygen and the release of one molecule of water, whereas the latter involves the uptake of only one molecule of oxygen with the release of one molecule of water. Thus the uptake of at least one molecule of oxygen is intimately concerned with the phytol-chlorophyllide link.

Before the exact chemical nature of this reaction can be elucidated, the solid reaction products will have to be analysed to determine the nature of the postulated products A, B and X. This can probably be done most readily by means of paper chromatography, and some preliminary experiments have been carried out. They have served at present only to demonstrate some of the complexities which may be met with in any analytical attempts; though the problem appears amenable to paper chromatography, a compound which was supposed to be inert to the attack of oxygen under these conditions showed six spots in place of the two standard spots. This tendency to breakdown of the molecule without an associated pressure change has been found before - spectra of extracted films of chlorophyll have shown the presence of phaeophytin, although a perfectly satisfactory unit ratio was obtained from the pressure curves

(i.e. phaeophytin free). This breakdown is probably due to the treatment of the sample on depositing and redissolving, and not due to undetected reactions involving pressure changes. Blank determinations have shown that the absorption spectrum of chlorophyll in acetone or ether is not affected by thallous bromide. The figures shown in the appendix (p.104) show that there is also a considerable decrease in the relative height of the red (6600 Å) absorption maximum, indicating loss of the two hydrogen atoms on ring IV.

Although the solid residue must be examined in detail before any rigid scheme for the mechanism can be put forward, it is interesting to speculate on the possible nature of the reaction. Firstly the reaction of phytol; the known oxidative degradations of the molecule (<u>in solution</u>) include (a) the oxidation of the double bond by chromic oxide or ozone to form a long chain ketone I and glycollic aldehyde (67), and (b) the formation of the acid II by oxidation via peroxide (68).



As films of phytol illuminated in oxygen have been shown to be acid after reaction, it would appear more likely that reaction (b) is taking place; this allowing for the uptake of one molecule of oxygen and the release of one molecule of water.

Thus it becomes possible that the irreproducible pressure ratios obtained with phytol are due to varying degrees of dissociation of the peroxide in equilibrium with water; as this compound dissociated, the amount of water released would increase, and consequently the pressure ratio would decrease. The extent of dissociation of the peroxide may depend on the conditions actually within the film, particularly the ease with which the resulting water could diffuse away. This reaction will require the presence of both peroxide and acid in the same film, and can be represented as:-

(1) Phytol + oxygen $\longrightarrow$ peroxide

(2) Peroxide $\rightleftharpoons$ acid + water.

It will be necessary to examine the oxidised film in greater detail for these postulated products to confirm this scheme.

The reaction of the intact chlorophyll molecule differs from that of phytol in the additional molecule of oxygen that is taken up. In addition, the above reactions of phytol are not likely to occur unchanged when the alcohol is esterified to the rest of the chlorophyll molecule. It is possible that

as the absorption spectra indicate a decrease in the relative height of the red (6600 Å) absorption peak, the additional molecule of oxygen is concerned with the removal of the two hydrogen atoms on ring IV of the porphyrin nucleus, possibly with the formation of one molecule of water. It is known that simpler chlorins may be photo-oxidised to porphins by molecular oxygen, i.e. the additional hydrogens in the saturated bond are removed (33). The formation of the peroxide on the double bond of the phytol group may still occur, but in this case it must be stable, as it has been previously found that there is a relatively stable peroxide group in oxidised films of chlorophyll. The method used in this work (p.38) for the detection of peroxide was not entirely successful, as only 30 - 50% of the total amount of peroxide previously revealed was detected. This is believed to be due to the large blank absorption of the potassium iodide and unreacted chlorophyll.

Thus the present work has made it possible to simplify the previous rather complicated expression for the photo-oxidation of solid chlorophyll, and has also indicated the most likely point of attack in the molecule.

Further work must confirm this simplification by re-examining the reaction of chlorophyll in the presence of phosphoric ~~acid~~ oxide; although irreproducible pressure ratios have

been obtained repeatedly in the past at values ranging from two to five, the present theory requires that the value be two. No difficulty has been experienced with the use of P_2O_5 in the examination of other compounds, (phaeophytin and phytol) and it therefore seems important that this aspect of the work should be investigated once again. Another point which should be confirmed is that pure ethyl chlorophyllide has indeed no reaction under these conditions; but the principal problem to the complete solution of this reaction is the analysis, and it is difficult to see how this may be overcome. The classical methods of chemical analysis are not, in general, sufficiently accurate to determine the composition and structure of the products of these reactions in the small quantities that are in use. Paper chromatography may have further applications, but will require the preparation and storage of a large number of the compounds suspected to be playing a part in this reaction. The analysis of phytol could not be readily attacked by this method, and it may be necessary to apply some of the more recent physical techniques, such as infra-red or ultra-violet spectroscopy.

APPENDIX

Decrease in the absorption of chlorophyll at 6600 Å after reaction:-

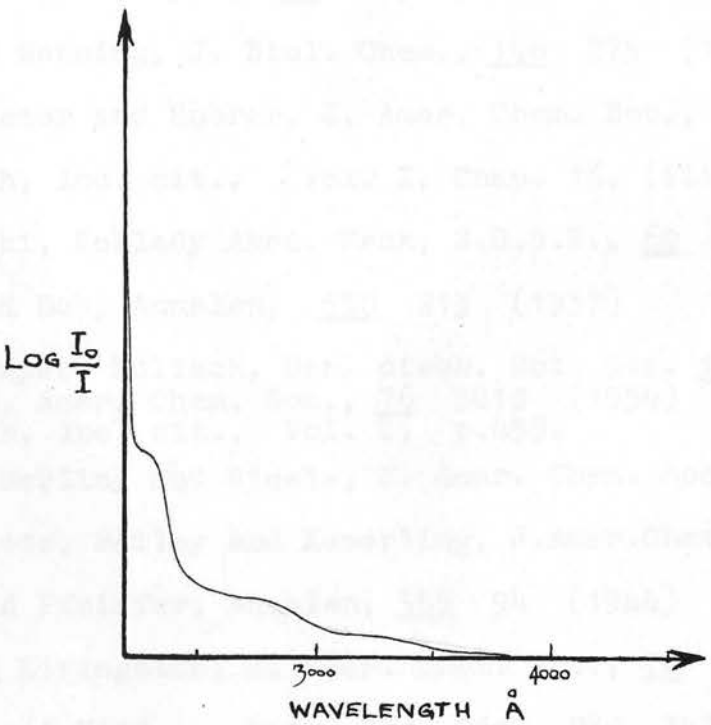
Run		Pressure Ratio.	$\frac{\Delta p}{\Delta p_o}$ x 100	Relative decrease at 6600 Å.
10	Chlorophyll	0.98	73	75%
11	Chlorophyll	0.95	78	80
14	Chlorophyll	0.94	29	58
15	Chlorophyll	0.99	39	50
16	Chlorophyll	1.01	25	46
25	Phaeophytin	3.08	93	71
26	Phaeophytin	3.10	83	85
28	Phaeophytin	2.98	75	94
29	Phaeophytin	3.04	66	74
30	Phaeophytin	3.17	77	74
52	Chlorophyll	1.04	88	58
57	Chlorophyll	0.98	48	50

Composition of Jena 'J' glass, used as substrate:-

SiO_2	69.0%
B_2O_3	7.0%
Fe_2O_3	0.2%
Al_2O_3	6.0%
ZnO	4.7%
BaO	5.5%
Na_2O	5.0%
K_2O	0.5%
Mn	0.04%
H_2O	0.3%

APPENDIX

Absorption spectrum of phytol in cyclohexane.



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